A DICER-LIKE PROTEIN IS ESSENTIAL FOR NORMAL SEXUAL DEVELOPMENT AND MEIOTIC SILENCING IN THE FILAMENTOUS FUNGUS *NEUROSPORA CRASSA*

A Thesis

by

MALCOLM THOMAS MCLAUGHLIN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2007

Major Subject: Biology

A DICER-LIKE PROTEIN IS ESSENTIAL FOR NORMAL SEXUAL DEVELOPMENT AND MEIOTIC SILENCING IN THE FILAMENTOUS FUNGUS *NEUROSPORA CRASSA*

A Thesis

by

MALCOLM THOMAS MCLAUGHLIN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee, Rodolfo Aramayo
Committee Members, James Erickson
Michael Polymenis
Debby Siegele
Head of Department, Vincent Cassone

December 2007

Major Subject: Biology

ABSTRACT

A Dicer-Like Protein Is Essential for Normal Sexual Development and Meiotic Silencing in the Filamentous Fungus *Neurospora crassa*. (December 2007)

Malcolm Thomas McLaughlin, B.A., University of Southern California; J.D., Chicago-Kent College of Law; B.S., Texas A&M University

Chair of Advisory Committee: Dr. Rodolfo Aramayo

The presence of an unpaired copy of a gene during meiosis triggers the silencing of every copy of that gene in the diploid ascus cell of *Neurospora crassa*, a phenomenon called Meiotic Silencing. This phenomenon has two stages: *trans*-sensing and meiotic silencing. If a DNA region is not detected on the opposite homologous chromosome early in meiosis (a *trans*-sensing failure), a signal corresponding to the unpaired region is produced that transiently silences expression of all homologous sequences. Meiotic silencing is related to RNA Silencing, a phenomenon that employs RNA-dependent RNA Polymerases (RdRPs), Argonautes, and Dicers. Dicers cleave double-stranded RNA (dsRNA) into 21-23 nucleotide RNAs. In the filamentous fungus *Neurospora crassa*, two RNA silencing pathways have been identified; one is active during mitosis, and the other is active during meiosis. The mitotic RNA silencing pathway, known as “quelling”, involves an RdRP (*quelling-deficient-1--qde-1*), an Argonaute-like protein (*quelling-deficient-2--qde-2*), and two Dicer-like proteins (*dicer-like-1*--

dcl-1 and *dicer-like-2--dcl-2*). Previous studies in *N. crassa* also revealed the involvement of an RdRP (*Suppressor of ascus dominance-1--Sad-1*) and an Argonaute-like protein (*Suppressor of meiotic silencing-2--Sms-2*) in meiotic silencing, suggesting that meiotic silencing is RNA-dependent and raising the question of whether a Dicer is involved in meiotic silencing.

In this work, we tested the participation in meiotic silencing of the *dcl-1* gene of *N. crassa,* which codes for a Dicer-like protein we call *Suppressor of meiotic silencing-3--Sms-3*. Crosses homozygous for mutant alleles of *Sms-3* are barren, indicating that the gene is also essential for sexual development. Due to this homozygous sterility, we could only test the involvement of *Sms-3* in meiotic silencing in heterozygous crosses. Under these conditions, we observed suppression of the meiotic silencing which would have otherwise been induced by the presence of unpaired DNA of reporter genes. We conclude that the Dicer-like protein *Sms-3* is required for both meiotic RNA silencing and sexual development.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION TO RNA SILENCING

Discovery of the RNA Silencing Phenomenon

Eukaryotic genomes efficiently regulate repeated elements. Compared to the percentage of human DNA coding strictly for proteins (~1.5%), at least 45% of the eukaryotic genome codes for transposable elements (HENIKOFF *et al.* 1997). Complex genomes must therefore evolve sophisticated ways to preserve identity and integrity in the presence of transposable elements and other insults. One of the most amazing such genome-defense mechanisms is RNA silencing, an ancient biological invention that can be considered to be equivalent to the immune system of the genome (COGONI and MACINO 2000; MEISTER and TUSCHL 2004; MELLO and CONTE 2004; PLASTERK 2002; WATERHOUSE *et al.* 2001; ZAMORE 2002). RNA silencing is initiated when double-stranded RNA (dsRNA) is converted into small interfering RNAs (siRNAs). These siRNAs then recruit the silencing machinery to destroy homologous RNAs, including cellular messenger RNAs (mRNAs), to "silence" the unwanted genetic material (MEISTER and TUSCHL 2004; MELLO and CONTE 2004). RNA silencing is highly efficient; a few dsRNA molecules can silence large amounts of mRNA (SIJEN *et al.* 2001).

This thesis follows the style and format of Genetics.

There is great interest among scientists in the mechanisms of RNA silencing, including a process called RNA Interference (RNAi). RNAi utilizes short double stranded RNA (dsRNA) to induce a directed form of gene silencing at the post-transcriptional level. When RNAi is activated, single-stranded RNA (ssRNA) is converted into dsRNA and processed into 21- to 23-nucleotide sequences called short interfering RNAs (siRNAs) (ELBASHIR *et al.*, 2001; ZAMORE *et al.*, 2000). Messenger RNAs (mRNAs) that have sequence homology to these siRNAs are predicted to physically interact with the siRNAs and to then be targeted for degradation (SAUNDERS and BARBER 2003). RNAi and other mechanisms of post-transcriptional gene silencing (PTGS) have been shown in a number of organisms, including *Drosophila melanogaster* (KENNERDELL and CARTHEW, 1998), *Caenorhabditis elegans* (FIRE *et al.*, 1998), *Danio rerio* (WARGELIUS *et al.*, 1999), and *Arabidopsis thaliana* (DEHIO and SCHELL, 1994; HAMILTON and BAULCOMBE, 1999).

It is appropriate to first recount the auspicious beginnings of the RNA silencing revolution. A phenomenon called Post-Transcriptional Gene Silencing (PTGS) was first observed in transgenic plants (NAPOLI *et al*., 1990; SMITH *et al*., 1990; VAN DER KROL *et al*., 1990). In an effort to enhance the color of petunia plants, Napoli *et al*. attempted to over-express the gene encoding the enzyme chalcone synthase (*chs*), which is responsible for the pigmentation of petunia flowers. Additional *chs* copies were introduced in an attempt to isolate darker flowers via

increased *chs* expression (NAPOLI *et al*., 1990). Surprisingly, the resulting flowers were white, similar to the *chs* mutant phenotype. Insertion of the exogenous copies suppressed color expression, rather than enhancing it; expression of all copies of the pigmentation gene appeared to be turned off by introduction of additional copies. This phenomenon was originally referred to as “co-suppression”. The molecular mechanisms driving this process were not understood at the time.

A similar PTGS phenomenon, referred to as “quelling”, was discovered in *Neurospora crassa* a few years later (ROMANO and MACINO, 1992). Romano and Macino sought to over-express the *albino-1* (*al-1*) gene responsible for the biosynthesis of carotenoids that give *N. crassa* its orange color. However, the introduction of extra *al-1* gene copies led to the production of a percentage of white progenies that were similar to the *al-1* mutant phenotype, a result similar to that obtained by Napoli (NAPOLI *et al*., 1990) in petunia plants. Extensive study has shown that proteins involved in PTGS in transgenic plants and quelling in *N. crassa* are evolutionarily conserved, but that each system has its own distinctive features (FULCI and MACINO, 2007).

Evidence to suggest that RNA may play a critical role in post-transcriptional gene silencing came in 1995, with the discovery of anti-sense RNA-mediated silencing (MELLO and CONTI, 2004). In the first published application of this

technique, large amounts of RNA complementary to the *abnormal embryonic PARtitioning of cytoplasm* (*par-1*) gene (either “control” sense or “experimental” antisense) were delivered into the cellular cytoplasm of *Caenorhabditis elegans* in an attempt to block *par-1* expression (GUO and KEMPHUES 1995). It was expected that anti-sense RNA would bind to *par-1* mRNAs and thus passively regulate expression of the gene, while the “control” sense RNA would not. It was discovered, however, that both sense and anti-sense RNAs, acting alone, induced silencing of *par-1* (GUO and KEMPHUES 1995). Obviously, the “control” sense RNAs could not pair with *par-1* mRNA. Clearly there was some *active* mechanism for RNA-induced silencing at work, induced by the presence of foreign RNAs.

In 1998, FIRE *et al.* reported that double-stranded RNA (dsRNA) produced a markedly stronger silencing effect than did either sense or anti-sense ssRNA, noting that only a few molecules of injected double-stranded RNA were required per cell, and suggesting the role of some catalytic amplification system (FIRE *et al.*, 1998). Furthermore, the silencing effect of these interfering dsRNAs was transmitted through the germ line from experimental worms to several generations of progeny, and the effect in the experimental animals often spread throughout tissues beyond the injection site (FIRE *et al.*, 1998; GRISHOK *et al.*, 2000). It was this discovery of dsRNA as a potent initial trigger for the system-

wide, heritable induction of silencing (FIRE *et al.*, 1998) that led to the adoption of the term RNA Interference (RNAi) (MELLO and CONTI, 2004).

Neurospora crassa has proven to be an excellent model system for the study of gene silencing. Studies in *N. crassa* have identified several distinct silencing mechanisms. A transcriptional gene silencing process, known as Repeat Induced Point Mutagenesis (RIP), occurs when DNA duplications are detected and populated with G:C to A:T mutations prior to meiosis in *N. crassa*; cytosine methylation is typically associated with sequences that are subjected to RIP (GALAGAN and SELKER, 2004; SELKER 2002; SINGER *et al*., 1995). Additionally, two distinct post-transcriptional gene silencing mechanisms have been identified in *N. crassa*, one induced during mitosis (quelling) and another induced during meiosis (Meiotic Silencing). Quelling occurs in *N. crassa* when the ectopic insertion of additional copies of a particular gene leads to a reduction in expression of all copies of that gene, endogenous and exogenous (COGONI *et al*., 1996; ROMANO and MACINO 1992). Meiotic Silencing occurs when a gene is not paired with its homolog in prophase I of meiosis; this unpaired region generates a signal that transiently silences all sequences homologous to it (LEE *et al.*, 2003b; SHIU and METZENBERG 2002). The presence of this unpaired DNA was proposed to activate an RNA-dependent silencing mechanism upon the discovery that mutations to an RNA-dependent RNA polymerase (RdRP) gene called *Suppressor of ascus dominance-1* (*Sad-1*) eliminate the silencing

otherwise induced by the presence of unpaired *Ascospore maturation-1* (*Asm-1*) and other genes (SHIU *et al*., 2001).

Components and Mechanisms of RNA Silencing

The independent discovery and study of RNA silencing mechanisms in *Arabodopsis thaliana, Caenorhabditis elegans, Drosophila melanogaster* and *Neurospora crassa* ultimately led to the realization that these organisms share similar underlying molecular mechanisms for RNA silencing (CATALANOTTO *et al.* 2000; COGONI and MACINO 1999b; COGONI and MACINO 2000; HUTVAGNER and ZAMORE 2002; MEISTER and TUSCHL 2004; MELLO and CONTE 2004; PLASTERK 2002; WATERHOUSE *et al.* 2001; ZAMORE 2002). The most important players in the RNA silencing mechanisms are the RNA-dependent RNA Polymerases (RdRPs), the Argonautes, and the Dicer proteins. To understand how these components work together to induce silencing, it is important to describe each component before discussing the interaction of each component in the silencing pathway.

RNA-Dependent RNA Polymerases (RdRPs) play an early role in the silencing pathway. RdRPs contain an RNA polymerase domain that converts single-stranded RNA (ssRNA) into double-stranded RNA (dsRNA), thus catalyzing the synthesis of RNA from an RNA template (MEISTER and TUSCHL 2004; MELLO and

CONTE 2004). The role of RdRPs in RNA silencing is proposed to be the synthesis of dsRNA from aberrant ssRNA as well as target messenger RNA (mRNA) of the same sequence (MELLO and CONTE 2004). It is suggested that RdRPs first recognize the presence of aberrant RNA sequences, and then convert the aRNA into dsRNA. The activity of RdRPs in generating dsRNA from mRNA is suggested to be the trigger for RNA silencing, with dsRNA acting as an early intermediary in the silencing pathway (MELLO and CONTE 2004). Genes encoding for RdRPs that participate in RNA silencing have been identified in a variety of organisms, including *SGS2* and *SGS3* in *A. thaliana* (MOURRAIN, P., *et al*., 2000; DALMAY, T., *et al*., 2000), EGO-1 in *C. elegans* (SMARDON, A.M. *et al* 2000), and *qde-1* (vegetative quelling pathway) and *Sad-1* (meiotic silencing pathway) in *N. crassa* (COGONI and MACINO 1999; SHIU *et al*., 2001). The role of RdRP in *N. crassa* is discussed later.

Dicer proteins also play a role in RNA silencing. Dicers are RNase III ribonucleases that cleave dsRNA into short (20-25 nucleotides) double-stranded RNA fragments called short interfering RNAs (siRNAs) (MACRAE *et al.*, 2006), by introducing either single-stranded nicks or double-stranded breaks (BERNSTEIN *et al*., 2001; SAUNDERS and BARBER, 2003). Common functional domains of the Dicers are catalytic RNase III domains, dsRNA-binding domains (dsRBDs), and RNA helicase (DEAD) domains (MEISTER and TUSCHL 2004; ARAVIND and KOONIN 2001; MIAN 1997; ROTONDO and FRENDEWEY 1996). The RBD allows

Dicers to bind to dsRNA, and the RNAse III domain catalyzes the cleavage of dsRNA into siRNAs. Some Dicers also have a Pinwheel-Argonaute-Zwille (PAZ) domain (MEISTER and TUSCHL 2004; BERNSTEIN et al., 2001; CERUTTI *et al.*, 2000), although this domain is absent from *N. crassa* Dicer-like proteins.

Dicers are highly conserved and have been identified in many organisms, including DCR-1 and DCR-2 in *D. melanogaster* (LEE *et al.*, 2004; PHAM *et al.* 2004; LIU *et al*. 2003; BERNSTEIN et al., 2001), DCR-1 in *C. elegans* (GRISHOK *et al.*, 2001; KNIGHT and BASS, 2001), DCL2 and DCL3 in *A. thaliana* (XIE, Z. *et al*., 2004; JACOBSEN *et al.*, 1999), and *dcl*-1 and *dcl*-2 in the vegetative quelling pathway of *N. crassa* (CATALONOTTO *et al*, 2004). In *D. melanogaster*, DCR-1 and DCR-2 processes dsRNA molecules in a stable association with a dsRBP (LEE *et al.* 2004; LIU *et al*. 2003; MEISTER and TUSCHL 2004; PHAM *et al.* 2004). In *A. thaliana*, DCL2 and DCL3 have functions related to siRNA processing in association with dsRBPs (BOUTET *et al.* 2003; XIE *et al.* 2003). The role of *dcl*-1 and *dcl*-2 in RNA silencing in *N. crassa* is discussed later.

Argonaute proteins also play a critical role in RNA silencing. The Argonautes are approximately 100 kDa proteins, conserved across many organisms, which contain two primary domains, a PAZ domain and a PIWI domain (MEISTER and TUSCHL 2004; CARMELL *et al*., 2002; CERUTTI *et al.*, 2000). The Argonaute PAZ domain generally consists of 130 amino acids, and has been identified in many

Argonaute proteins and also in some Dicer proteins (MEISTER and TUSCHL 2004; CARMELL et al., 2002). The PAZ domain was initially suggested to be involved in a protein-protein interaction between Argonaute and Dicer, because the domain is found in both Argonautes and Dicers and also because Argonautes have been found to co-immunoprecipitate with Dicers (MEISTER and TUSCHL 2004; CARMELL *et al*., 2002; HAMMOND *et al*., 2001). It has been suggested more recently, however, that the PAZ domain is in fact an RBD that recognizes and binds to siRNA duplexes (MEISTER and TUSCHL 2004; MA, J.B. *et al*., 2004; LINGEL, A. *et al*., 2004), presumably generated by the catalytic activity of Dicers on dsRNA. Thus, the PAZ domain of Argonaute proteins likely binds to the siRNA products that are generated by the cleavage activity of Dicer on dsRNA. The function of PIWI domains in Argonaute is not known (CARMELL *et al.*, 2002).

Argonautes are also highly conserved across model organisms, and the number of Argonautes present varies from organism to organism. Four Argonaute proteins have been identified in *D. melanogaster* (Piwi, Aubergine, dAgo1 and dAgo2), each of which is implicated in RNA silencing (MEISTER and TUSCHL 2004; CARMELL *et al*., 2002; MARTINEZ *et al*., 2002; WILLIAMS and RUBIN, 2002; PAL-BHADRA M., *et al*., 2002; KENNERDELL, J.R., et al., 2002; HAMMOND *et al*., 2001). In *C. elegans*, RDE-1 and PPW-1 are implicated in efficient RNAi activity (MEISTER and TUSCHL 2004; CARMELL *et al*., 2002; TABARA, H. *et al*., 2002; TIJSTERMAN, M. *et al*., 2002; FAGARD, M. *et al*., 2000). In *A. thaliana*, AGO-1 is

involved in post-transcriptional gene silencing, the RNA silencing mechanism first observed in plants (CARMELL *et al*., 2002; FAGARD, M. *et al*., 2000). Argonaute-like proteins are involved in both the vegetative quelling (QDE-2) and meiotic silencing pathways (SMS-2) of *N. crassa* (LEE *et al*., 2003b; FAGARD, M. *et al*., 2000; COGONI and MACINO 1997).The roles of *qde*-2 and *sms*-2, the genes that encode for QDE-2 and SMS-2 in *N. crassa* are discussed later.

These three components of RNA silencing are suggested to act in an orderly pathway that leads to the destruction of any mRNA with sequence homology to the aberrant RNA that initiated the pathway. This RNA silencing model suggests that: (1) aberrant ssRNA is converted into dsRNA by an RdRP and cleaved into double stranded siRNA by Dicer; (2) the siRNA is bound to an RNA-induced silencing complex (RISC) by an Argonaute and then unwound to single-stranded siRNA by an RNA helicase; and (3) the RISC complex targets for destruction mRNAs that share sequence homology with siRNAs bound up in the complex.

RISC was first identified in *D. melanogaster*, where biochemical analyses suggested that the *Drosophila* RISC nuclease is a ribonucleoprotein requiring both RNA and protein components, and that the components of RISC are likely in association prior to binding homologous mRNAs, rather than being assembled on the target mRNA after acquisition. (HAMMOND, S.M. *et al*., 2000). Gel electrophoresis assays later showed that RISC assembly is a three step process

involving an initiator (R1), an intermediate (R2), and an effector (R3) known as holo-RISC (PHAM, J.W. and E.J. SONTHEIMER, 2005). The R1 initiator is formed by association of a Dicer-2/R2D2 heterodimer (R2D2 is a dsRBP) with siRNAs; this heterodimer is sufficient to initiate RISC formation. The initiator is then converted into an intermediary (R2), and Argonaute 2 is ultimately recruited to form the R3 holo-RISC (PHAM, J.W. and E.J. SONTHEIMER, 2005). RISC activity is also suggested as the basic mechanism for RNA silencing in *A. thaliana*, *S. pombe* and *C. elegans* as well (MEISTER and TUSCHL 2004). The assembly of the RISC complex is ATP-dependent (NYKANEN *et al.* 2001; PHAM *et al.* 2004), which likely indicates a need for energy-driven unwinding of double-stranded siRNAs in the RISC machinery (MEISTER and TUSCHL 2004).

Silencing in *Neurospora crassa*

N. crassa employs two different pathways for post-transcriptional gene silencing, one of which is vegetative silencing or quelling. A number of genes have been identified that are central to quelling. These genes are: *quelling deficient-1(qde-1)*, which encodes for an RdRP; *quelling deficient*-2 (*qde-2*), which encodes for an Argonaute-like protein: *quelling deficient*-3 (*qde-3*), which encodes for a Rec-Q DNA helicase; and *dicer-like*-1 (*dcl-1*) and *dicer-like-2* (*dcl-2*), which encode for Dicer proteins (CATALONOTTO *et al.*, 2004; CATALONOTTO *et al.*, 2002; COGONI and MACINO, 1999a). A functional copy of either *dcl-1* or *dcl-2* alone is required for quelling, since only a *dcl-1*/*dcl-2* double mutant is defective in

quelling (CATALONOTTO *et al.*, 2004). Thus, *dcl-1* and *dcl-2* are redundant in the quelling pathway. Another form of post-transcriptional gene silencing occurs in *N. crassa* during prophase 1 of meiosis. To date, two important genes have been identified in this pathway; these are *Suppressor of ascus dominance-1* (*Sad-1*), which encodes for an RdRP, and *Suppressor of meiotic silencing-2* (*Sms-2*), which encodes for an Argonaute-like protein (LEE *et al.*, 2003b; SHIU *et al.*, 2001). The involvement of an RdRP and Argonaute in both quelling and meiotic RNA silencing suggests a role for Dicer in each pathway as well.

To study the role of Dicer in the meiotic RNA silencing mechanism of *N crassa*, one must first understand the connection between meiotic RNA silencing and meiotic *trans*-sensing. Meiotic *trans*-sensing is the process by which *N. crassa* chromosomes sense each other and align properly during meiosis (ARAMAYO and METZENBERG, 1996). Meiotic *trans*-sensing in *Neurospora* was discovered as a result of the study of the *Ascospore maturation-1* (*Asm-1*) locus. *Asm-1* is the locus responsible for the development of mature sexual ascospores upon completion of meiosis. Mutations to *Asm-1* block the production of healthy ascospores in an ascus-dominant fashion (ARAMAYO and METZENBERG, 1996; ARAMAYO *et al.*, 1996). Additionally, the introduction of an extra, unpaired copy of *Asm*-1 into an otherwise wild-type cross similarly blocks the production of healthy spores; crosses in which an unpaired *Asm-1* locus is introduced produce only white, dead ascospores even in the presence of two functional *Asm-1*$^{+}$

alleles (ARAMAYO and METZENBERG, 1996; ARAMAYO *et al.*, 1996) (Figure 1). Thus, unpaired regions of DNA appear to cause a failure in trans-sensing during meiotic transvection and induce the silencing of *Asm-1*. These results are consistent with the notion that the presence of an unpaired locus will destroy the functionality of that gene at every chromosomal locus during meiosis.

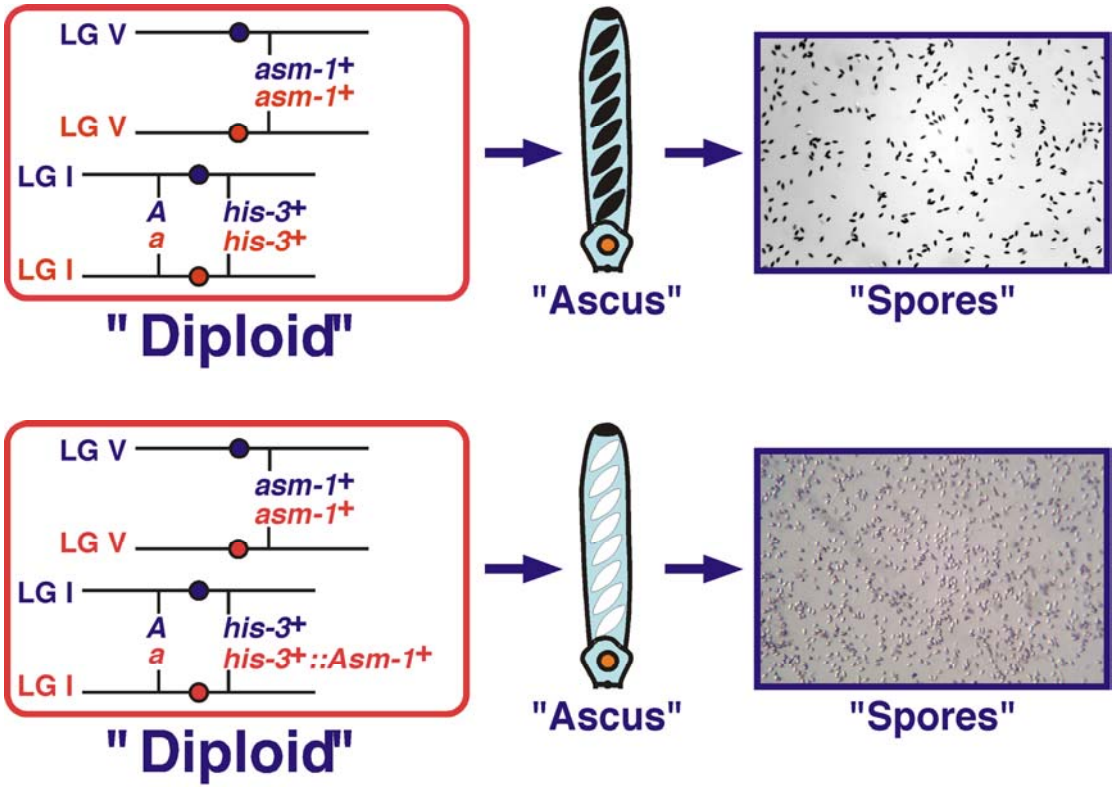


Figure 1 - Crosses with unpaired *Asm-1*$^+$ yield nonviable progenies. Crosses with two wild type strains yield viable, black ascospores due to the presence of two functional copies of *Ascospore maturation-1* (*ams-1*$^+$). However, if a fragment of unpaired *Asm-1*$^+$ DNA is inserted ectopically (at *his-3*$^+$) into an otherwise wild type strain and that strain is crossed to a wild type strain, the resulting progeny are white, nonviable ascospores. The insertion of unpaired DNA thus appears to induce silencing even in the presence of two functional *asm-1*$^+$ alleles.

LG = chromosomal linkage group; A/a = mating type; *asm-1*$^+$ = wild-type *Ascospore maturation-1* gene responsible for ascus maturation and viable spores; *his-3*$^+$ = histidine, locus of unpaired *Asm-1*$^+$ insertion.

As previously noted, there are two distinct post-transcriptional gene silencing pathways in *N. crassa*, quelling and Meiotic RNA Silencing. The RNA dependent nature of silencing in the quelling pathway is established. An RdRP (*qde-1*) and an Argonaute-like protein (*qde-2*) were identified as components of quelling in 1997 (COGONI and MACINO 1997). Additionally, mutations to the gene encoding for *Suppressor of ascus dominance-1* (*Sad-1*), a *qde-2* paralog, were shown to suppress the silencing otherwise induced by introducing unpaired *Asm-1*, the gene responsible for ascospore maturation (SHIU *et al.*, 2001). On the basis of these observations, it was proposed that the presence of unpaired chromosomal regions lead to the activation of an RNA-mediated silencing pathway.

Similarly, evidence for RNA dependent meiotic silencing in *N. crassa* has also been shown. Scanning of the *N. crassa* genome revealed the existence of a *qde-2* paralog called *Suppressor of meiotic silencing-2* (*Sms-2*) which encodes for an Argonaute-like protein, suggesting involvement in the meiotic silencing pathway (LEE et al., 2003b). Strains containing mutant *Sms-2* alleles were generated via Repeat Induced Point Mutation (RIP). Homozygous crosses of the *Sms-2* RIP strains are barren, however, requiring crosses heterozygous for *Sms-2*. To test the involvement of *Sms-2* in meiotic silencing, strains with mutant *Sms-2* RIP alleles were crossed to strains with ectopically inserted unpaired *Asm-1* DNA; all crosses were homozygous wild-type *asm-1*$^{+}$ at the canonical

locus and heterozygous for *Sms-2*. It was predicted that the *Sms-2* RIP alleles would be unable to pair with the *sms-2*$^{+}$ wild-type alleles, which would induce a partial silencing of the *sms-2*$^{+}$ allele itself, leading to a reduction in the amount of available *sms-2*$^{+}$ transcript. It was further predicted that, if *sms-2*$^{+}$ is involved in meiotic silencing, a reduction in *sms-2*$^{+}$ transcript would lead to a less functional meiotic silencing system and a population of viable ascospores, even in the presence of unpaired *Asm-1* DNA. The results of the crosses were a population of viable ascospores, confirming the hypothesis that the Argonaute-like protein encoded for by *sms-2*$^{+}$ is involved in meiotic silencing (LEE *et al.*, 2003b).

Viable ascospores were also observed in heterozygous crosses between wild-type *sad-1*$^{+}$ and mutant *Sad-1* RIP strains in the presence of unpaired *Asm-1* DNA (LEE *et al.*, 2003b), indicating that *sad-1*$^{+}$ is also involved in the meiotic silencing pathway and that a failure in *sad-1*$^{+}$ pairing leads to reduced silencing efficiency. Lee *et al.* proposed a model in which the failure of chromosomal pairing serves as a trigger for the induction of aberrant RNA (aRNA), which is converted into dsRNA by a SAD-1 RdRP. It is suggested that the dsRNAs are converted into siRNAs by a Dicer-like endonuclease, at which point siRNAs are fed into a SAD-1-mediated amplification cycle and an SMS-2-mediated effector cycle to target mRNAs from the unpaired DNA for degradation (LEE *et al.*, 2003).

The involvement of an RdRP (*sad-1*$^{+}$) and an Argonaute (*sms-2*$^{+}$) in the meiotic silencing pathway strongly suggest that meiotic silencing in *N. crassa* is RNA dependent, and may involve a role for Dicer. The role of Dicers in RNA Silencing has been well studied in the vegetative quelling pathway. In quelling, *dcl*-1 and *dcl*-2 convert dsRNAs into ~21 nucleotide siRNAs (CATALONOTTO *et al*., 2004). Protein extracts from a *dcl-1*/*dcl-2* double mutant fail to process dsRNAs into ~21 nucleotide siRNAs (CATALONOTTO *et al*., 2004), implying that conversion of dsRNAs into siRNAs is an intermediate step in quelling.

The role of Dicer in meiotic silencing in *N. crassa* is currently a subject of study. The presence of an RdRP, an Argonaute-like protein, and two Dicers in the quelling pathway, combined with the identification of an RdRP (*Sad-1*) and an Argonaute-like protein (*Sms-2*) in meiotic RNA silencing, strongly suggests a role for Dicer in the meiotic silencing system. At the time of this work, no discoveries had been made regarding the role of Dicer in meiotic RNA silencing in *N. crassa*. It is my hypothesis that *N. crassa* utilizes one or more Dicer-like proteins in the meiotic silencing pathway to cleave dsRNAs into siRNAs, and that Dicer is one component of an RNA-mediated meiotic silencing complex. The identification and characterization of a Dicer-like activity in the meiotic silencing pathway of *Neurospora* is therefore the focus of this work.

CHAPTER II

sms-3^{+} IS ESSENTIAL FOR NORMAL SEXUAL DEVELOPMENT AND EFFICIENT MEIOTIC SILENCING IN *NEUROSPORA CRASSA*

Introduction

The filamentous fungus *N. crassa* distinguishes itself from other organisms by having several, sometimes interconnected genome defense mechanisms: DNA methylation, vegetative RNA silencing (quelling), Repeat Induced Point mutation (RIP) and meiotic silencing (BORKOVICH *et al.* 2004; GALAGAN *et al.* 2003). Although the vegetative and the meiotic RNA silencing pathways share the same objective (i.e., preserve genome identity and integrity), they have evolved to defend the organism at two very different developmental stages, haploid and diploid, respectively. Haploid-specific RNA silencing (i.e., quelling) senses the presence of repeated elements in the genome, probably by measuring the level of aberrant-RNA (aRNA), and reacts by recruiting RNA-dependent RNA polymerases (RdRPs), Argonaute-like proteins, and Dicer-like proteins to initiate the production of siRNAs (CHICAS *et al*., 2004; FORREST *et al*., 2004; GOLDONI *et al*., 2004; CATALANOTTO *et al*., 2004; CATALANOTTO *et al*., 2002; PICKFORD *et al*., 2002; COGONI *et al*., 2001; COGONI 2001; COGONI and MACINO, 1999a; COGONI and MACINO 1999b; COGONI and MACINO 1999c). The diffusible siRNAs, in turn, act by interfering with the propagation of repeated elements within and between

sibling nuclei by directing the destruction of any messenger RNA (mRNA) with homology to the siRNAs (COGONI *et al.* 1996).

In contrast, the meiotic silencing pathway is only activated in cells undergoing meiosis (ARAMAYO and METZENBERG 1996b; SHIU *et al.* 2001). In *Neurospora*, meiosis occurs inside an ascus-mother cell called the zygote, a cell that initially contains two haploid nuclei of opposite mating type (RAJU 1980; RAJU 1992). The zygote develops into an ascus cell inside the perithecium, the complex multicellular sexual reproductive apparatus of Neurospora that carries up to 200 developing asci immersed in maternal tissue. The nuclei inside the zygote undergo karyogamy, meiosis, post-meiotic mitosis, and are compartmentalized resulting in an ascus cell that contains eight haploid spores (i.e., ascospores) arrayed in an order that reflects the cell's lineage (RAJU 1980; RAJU 1992). Immediately following karyogamy, the genomes of the diploid zygote cells are scrutinized by meiotic *trans*-sensing, a process that evaluates symmetry between the chromosomes participating in meiosis (ARAMAYO and METZENBERG 1996b). When an insertion, a deletion, or a homeologous region is detected on the opposite chromosome, *trans*-sensing is affected, a situation that directly results in the activation of meiotic RNA silencing (ARAMAYO and METZENBERG 1996b; SHIU *et al.* 2001). It has been hypothesized that meiotic RNA silencing then uses the region of unpaired DNA as a template to produce an RNA-based diffusible signal which silences the expression of all genes with homology to the

ones present in the loop of unpaired DNA, regardless of their pairing (LEE *et al.* 2003b; SHIU *et al.* 2001). It follows that, if the corresponding gene product is required for the later stages of meiosis or ascospore development, then the destruction of its mRNA may abort meiosis, block ascospore maturation or result in the production of a crop of morphologically abnormal ascospores (ARAMAYO and METZENBERG 1996b; ARAMAYO *et al.* 1996; LEE *et al.* 2003b; LEE *et al.* 2004; SHIU and METZENBERG 2002; SHIU *et al.* 2001). Once activated, meiotic silencing persists during the subsequent meiotic divisions, but it is reset at some point prior to ascospore germination.

Detecting meiotic silencing is challenging, and requires the use of well-defined reporter genes. According to the prevailing model, the presence of unpaired DNA *per se* induces meiotic silencing, but its detection is dependent on the ability of the genes present in the loop of unpaired DNA to affect either the later stages of meiosis or spore development. In this work we have used two reporter genes: *Ascospore maturation-1* (*Asm-1*) and *Roundspore* (*Rsp*). The product of *asm-1*$^{+}$(ASM-1) is an abundant nuclear protein essential for the formation of aerial hyphae, the development of protoperithecia (the haploid female sexual structures), and the maturation of the ascospores (the haploid sexual spores) (ARAMAYO and METZENBERG 1996b; ARAMAYO *et al.* 1996). Spores carrying recessive, loss-of-function mutations in *Asm-1* fail to develop, whereas spores with the *asm-1*$^{+}$ allele mature normally (ARAMAYO and METZENBERG 1996b;

ARAMAYO *et al.* 1996). Unpairing of *Asm-1* in meiosis, via the insertion of an ectopic copy of *Asm-1*, results in a progeny of dead (i.e., white) ascospores (ARAMAYO and METZENBERG 1996b; ARAMAYO *et al.* 1996; KUTIL *et al.* 2003; LEE *et al.* 2003b; LEE *et al.* 2004; SHIU *et al.* 2001) (Figure 1). In contrast, the product of *rsp*$^{+}$ (RSP) is required for the formation of spindle- or football-shaped ascospores, but is not required for viability (SRB *et al.* 1973). Unpairing of *Rsp* in meiosis, via the introduction of a RIP allele into the cross, results in a progeny of spores with round or ovoid morphology (PRATT *et al.* 2004; SHIU *et al.* 2001).

Unpairing of either one of these two reporter genes, or of any other reporter gene, can result in either *cis*- or *trans*-silencing (LEE *et al*., 2004; SHIU *et al*., 2001). If an unpaired gene is not involved in meiotic silencing, then unpairing of that gene via insertion of an ectopic copy will not affect the silencing pathway. If, however, the unpaired gene is involved in meiotic silencing, its own silencing via an ectopic duplication should lead to a level of suppression of the silencing mechanism - via the silencing of that component. These alternative outcomes are represented in Figure 2, in the context of unpaired Dicer-like genes *sms*-3 and *dcl*-2 (discussed in detail in the Results section below). If a transcribed region is included in the loop of unpaired DNA, the region is *cis*-silenced. If, during *cis*-silencing, paired regions homologous to the loop of unpaired DNA are present elsewhere in the genome, then the diffusible signal produced by the loop of unpaired DNA will *trans*-silence their expression.

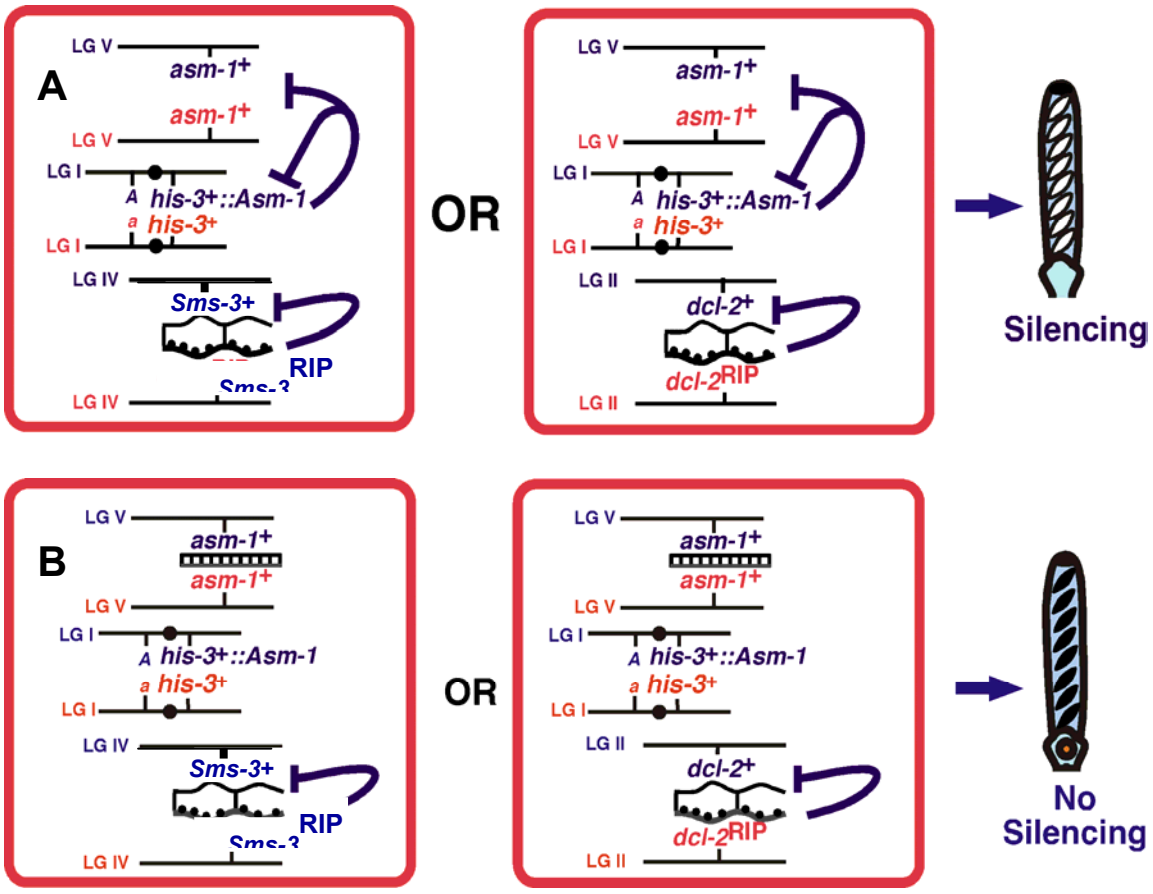


Figure 2 – Unpairing of a Dicer-like gene may interfere with silencing. If neither *sms-3* nor *dcl-2* is involved in the meiotic silencing pathway (A), then the resulting progeny will be white, nonviable ascospores due to the silencing induced by unpaired *Asm*-1 DNA. However, if either Dicer-like gene is involved in meiotic silencing (B), crosses between a strain carrying unpaired *Asm-1* or *Rsp* DNA and another strain carrying a RIP allele of the gene(s) will yield a population of black, viable ascospores due to suppression of meiotic silencing.

LG = chromosomal linkage group; A/a = mating type; *asm-1*$^{+}$ = wild-type *Ascospore maturation-1* gene responsible for maturation of ascus and production of viable (black) spores; *his-3*$^{+}$ = histidine, the locus of unpaired *Asm-1*$^{+}$ insertion.

Neurospora crassa is the organism of choice to study the interactions between RNA silencing pathways since it has two distinct pathways (BORKOVICH *et al.*,

2004; GALAGAN *et al*., 2003). Within the quelling pathway several genes, some of them homologous to RNAi genes in other systems, have been identified. These are: *quelling deficient-1* (*qde-1*), an RdRP (COGONI and MACINO 1999a); *qde-2*, an Argonaute-like protein (CATALANOTTO *et al.* 2000); *qde-3*, a RecQ DNA helicase (COGONI and MACINO 1999c); and the Dicer homologues *dicer like-1* (*dcl-1*) and *dicer like-2* (*dcl-2*) (BORKOVICH *et al.* 2004; CATALANOTTO *et al.* 2004; GALAGAN *et al.* 2003). Both *dcl-1* and *dcl-2* convert dsRNAs into ~21 nucleotide siRNAs, implying that the conversion of dsRNAs into siRNAs is an intermediate step in the quelling pathway (CATALANOTTO *et al.* 2004). Unlike *qde-1, qde-2* and *qde-3*, whose gene products are essential for silencing, the products of *dcl-1* and *dcl-2* are redundant, since only a *dcl-1*, *dcl-2* double-mutant is defective in quelling (CATALANOTTO *et al.* 2004).

The meiotic silencing pathway also requires the activity of an RdRP (*Suppressor of ascus dominance-1*, *Sad-1*) (LEE *et al.* 2003b; SHIU *et al.* 2001) and an Argonaute-like protein (*Suppressor of meiotic silencing-2*, *Sms-2*) (LEE *et al.* 2003b). The identification of an RdRP and an Argonaute suggests that meiotic silencing employs a mechanism similar to quelling and to RNAi pathways in other organisms. This hypothesis would be supported by the discovery of a Dicer-like protein involved in meiotic silencing. We tested this hypothesis and report that the product of *Suppressor of meiotic silencing-3*, the Dicer-like protein SMS-3, is required for sexual development and meiotic RNA silencing.

Materials and Methods

Procedures for Southern blot analysis and other nucleic acid manipulations were performed as described (PRATT and ARAMAYO 2002). Similarly, *N. crassa* growth conditions, conidial spheroplast preparation and fungal transformation were performed as described (PRATT and ARAMAYO 2002). Homokaryon purification was performed as described (LEE *et al.* 2003a; PRATT and ARAMAYO 2002). The formulas for the Vogel's Medium N, the Westergaard's Medium, and the sugar mixture of Brockman and de Serres have been described by Davis and de Serres (1970).

Neurospora genetic crosses—Set up and scoring. Mutant alleles of each Dicer-like gene were constructed, and their ability to suppress meiotic silencing was tested in a series of heterozygous crosses. Construction of the strains employed Repeat-Induced Point (RIP) mutagenesis. RIP mutations occur prior to meiosis if a sequence of DNA is present in more than one copy in a haploid genome (SELKER, 1990), such as when an unpaired *Asm-1* fragment is introduced at an ectopic locus. Upon crossing this duplication strain to another strain, GC-to-AT mutations are introduced into each copy of the sequence on the duplication strain. The RIP'd strain is then crossed to a wild-type strain to obtain a progeny RIP'd at the allelic locus but that is free of duplicated DNA. The length of a RIP'd region depends upon the length of duplicated DNA introduced.

Construction of each mutant Dicer-like allele was achieved via integration of a duplication of the particular gene at the *histidine-3* (*his-3*) locus on linkage group I (LG I), followed by crosses to yield the desired RIP'd alleles. Sequencing of each RIP'd allele was performed to confirm the presence of GC-to-AT transition mutations. Once strains carrying RIP'd alleles of each gene were identified, they were crossed to strains carrying unpaired *Asm*-1 DNA (which induces meiotic silencing of *asm*-1^{+} to produce white ascospores) and to strains carrying the *Rsp*^{RIP93} allele (which induces meiotic silencing of the *roundspore*$^{+}$ gene to produce round ascospores), in order to determine if mutations to *sms-3* or *dcl-2* suppress the meiotic silencing otherwise induced by the failure of chromosomal pairing of *Asm*-1 and by mutation of the Rsp^{RIP93} allele.

Scoring of silencing and suppression was conducted by counting the total percentages of white vs. black and round vs. spindle ascospores produced from each experimental cross, and comparing those data to the percentages of white vs. black and round vs. spindle ascospores produced in control crosses lacking mutant *Sms-3* or *dcl-2* alleles.

Partners were co-inoculated in a Petri dish containing Westergaard's medium, and incubated at 25°C for six to eight days. The two co-inoculated strains usually meet after day three to five. Excess conidia were removed only when

fertilization was evident. Crosses started shooting ascospores approximately 14 days after inoculation. Spores were harvested no sooner than 25 days after point inoculation. When one of the crossing partners carried the *fluffy* (*fl*) mutation, conidia from the *fl*$^{+}$ partner were used as males, fertilizing the female structures in the lawn of *fl* strains.

Importantly, *Rsp* mutants ooze most of their progeny through the perithecial ostiole. The remaining spores are weakly shot and rarely reach the lid of the Petri Dish. Therefore, to accurately assess the degree of silencing, spores must be collected from both the lids and the surfaces of the Petri Dishes. For this, we flooded the plate with sterile water and scrapped the perithecia off the surface of the agar with a sterile glass rod. The slurry of spores and tissue was then used to harvest the spores from the lid of the plate. The combined slurry was then transferred into a centrifuge tube. The process was repeated; this time washing both the front and back of the agar. After centrifugation, the supernatant was carefully removed by aspiration and discarded. Aliquots of the ascospore suspension were transferred onto glass microscope slides and under a cover slip. The degree of silencing was determined by taking nine pictures from different random fields (11X magnification + 115 mm zoom). Pictures were printed and the number of round and spindle-shaped spores was determined. The strength of the observed silencing was defined as by the percentage of black spindle-shaped ascospores: 100% = no silencing and 0% = total silencing.

While we observe that crosses between the same parents vary in their absolute number of wild-type ascospores produced (within ± 10% range), the ratio of wild-type ascospores produced by a given allele (e.g. *Sms-3*^{RIP65}) relative to another allele (e.g. *Sms-3*^{RIP19}) remains relatively constant within the same experiment. Why this variability occurs is not clear, but we believe it is due to the contribution of several, perhaps overlapping, factors such as temperature, oxygen availability, and illumination. If any or all these factors directly or indirectly influence DNA methylation, then they would likely also influence the dominant behavior of the RIP-allele in question (PRATT *et al.* 2004). This is why all statements regarding the behavior of one particular allele compared to another allele have been made based on data obtained from crosses that were set up and harvested at the same time and under the same conditions.

Oligonucleotides. The Oligonucleotides used in this study are described in Table 1 below:

TABLE 1 - Oligonucleotides used in this study

Name	Sequence
ODLAM220	5'-CACCATGGCCGTAGCCACTCGGCTACCCTTTATCC-3'
ODLAM221	5'-CTAAACCGCCGTCGGATGTACCTCTCCATCCAC-3'
ODLAM322	5'-AGGGGATCCTGGCAATGCTTCTCTGTTTC-3'
ODLAM323	5'-AGAGTCGACCCCAACCTCTCCCTGAACTC-3'
ODLAM324	5'-AGGGGATCCCCCAAGTCCAGCCAGTTCAA-3'
ODLAM325	5'-AGAGTCGACCCGTGTTATGCCCCTTATCC-3'
OMM003	5'-CCCTTCGTTGCTCCTCACATCCATTCA-3'
OMM004	5'-CCCGCTGCAATGCTGGGTATGTGAGAC-3'
OMM012	5'-CCCCTGCAGACTCCCCACCAACAGCAAGA-3'
OMM013	5'-CCCGATATCGCCAAGCACCTCACCAAAAT-3'
OMM014	5'-CCCCTGCAGTTCTGGTGGGAGCCGTTGGA-3'
OMM015	5'-CCCGATATCCTGGGGGAGGAGGGGATGGT-3'
OMM016	5'-GACGACCTCGCACTGA-3'
OMM017	5'-GATTCGATCTCTATGA-3'
OMM018	5'-ACCCTTGCCGCTTCTA-3'
OMM019	5'-CTCGGCTACCCTTTAT-3'
RIIIH2D	5'-AGGGGATCCCCGTGAGAGGTGTTGTGAGAAG-3'
RIIIH2U	5'-AGGGGATCCCAAGCCCGCTAAAGTCAAATAA-3'
RIIIH1D	5'-AGGGGATCCCTAAGCAGGAGAAGAGGACGAG-3'
RIIIH1U	5'-AGGGGATCCTGTTGTTACGCTGGGTCATTAC-3'

Plasmid construction. The genome sequence of the *Sms-3* locus is contained on contig 6.53 (Release 6, Whitehead Institute, http://www.genome.wi.mit.edu/annotation/fungi/neurospora/). In the region, we defined as position 1 the *Bam*HI site, located 982 bp upstream of the translational initiation signal (ATG) for SMS-3. Following this convention, the TAG codon (Stop) is located at position 5,850. Details about the *Asm-1*and the *Rsp* loci have been given in (KUTIL *et al.* 2003; LEE *et al.* 2003b; LEE *et al.* 2004; PRATT *et al.* 2004). The GenBank Accession Number corresponding to the *sms-3*^{RIP19} and *Sms-3*^{RIP65} alleles are AY596280 and AY262828, respectively. The following plasmids were constructed following standard procedures (AUSUBEL *et al.* 1987; SAMBROOK *et al.* 1989):

Plasmid pDCL-2. Constructed by first PCR-amplifying a 2,402 bp *dcl-2* fragment using RIIIH2U and RIIIH2D (Table 1) as primers, and DNA from RANCR06A, as a template. The PCR product was then digested with *Bam*HI and cloned into the *Bam*HI site of pRAUW122 (ARAMAYO and METZENBERG 1996a).

Plasmid pDLAM110a. Constructed by first PCR-amplifying a 4,869 bp *Sms-3* fragment (coordinates 982 to 5850) using ODLAM220 and ODLAM221 (Table 1) as primers, and DNA from RANCR06A, as a template. The PCR product was TOPO-cloned into pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA).

Plasmid pDLAM178. Constructed by first PCR-amplifying the 1,568 bp *Bam*HI-*Sal*I *Sms-3* fragment (coordinates 4196 to 5762) using ODLAM322 and ODLAM323 (Table 1) as primers, and DNA from RANCR06A, as a template. The PCR fragment was digested with *Bam*HI and *Sal*I and then cloned into the *Bam*HI-*Sal*I sites of pGEM3Zf(+) (Promega, Madison, Wisconsin, USA).

Plasmid pDLAM179. Constructed by first PCR-amplifying the 1,147 bp *Bam*HI-*Sal*I *dcl-2* fragment (coordinates 3949 to 5095) using ODLAM324 and ODLAM325 (Table 1) as primers, and DNA from RANCR06A, as a template. The PCR product was cloned into the *Bam*HI-*Sal*I sites of pGEM3Zf(+) (Promega). The anti-sense strand of *dcl-2* can be transcribed under the SP6 promoter.

Plasmid pDLAM183. Constructed by cloning the 446 bp *Eco*RI-*Nsi*I *Sms-3* fragment (coordinates 4987 to 5432) from pDLAM178 into the *Eco*RI-*Pst*I sites of pGEM3Zf(+) (Promega). The anti-sense strand of *Sms-3* can be transcribed under the SP6 promoter.

Plasmid pMMAM06. Constructed by first PCR-amplifying a 3,309 bp *Sms-3* fragment (coordinates -767 to 2542) using OMM003 and OMM004 (Table 1) as primers, and DNA from RANCR06A, as a template. The PCR product was

digested with *Eco*RI-*Xba*I and then cloned into the *Eco*RI-*Xba*I sites of pJHAM04 (HAAG *et al.* 2003).

Plasmid pMMAM18. The 4,869 bp *Sms-3* insert fragment (coordinates 982 to 5850) from pDLAM110a was transferred to pJHAM07 (HAAG *et al.* 2003) following standard Gateway™ cloning protocols (Invitrogen).

Plasmid pMMAM19. Constructed by first PCR-amplifying a 7,331 bp *Sms-3*^{RIP19} fragment (coordinates -158 to 7174) using OMM014 and OMM015 (Table 1) as primers, and DNA from MMNCR19A, as a template. A second 5,617 bp *Sms-3*^{RIP19} fragment (coordinates 406 to 6022) was PCR-amplified using OMM012 and OMM013 (Table 1) as primers, and DNA corresponding to the 7,331 bp *Sms-3*^{RIP19} fragment (the product of the first PCR amplification) as a template. This fragment was then digested with *Eco*RV and *Pst*I and cloned into the *Eco*RV and *Pst*I sites of pBSK+ (Stratagene, La Jolla, CA, USA) to generate pMMAM19.

Plasmid pSMS-3. Constructed by PCR amplifying a 1,945 bp *Sms-3* fragment (coordinates 1504 to 3449) using RIIIH1U and RIIIH1D (Table 1) as primers, and DNA from RANCR06A, as a template. The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of pRAUW122 (ARAMAYO and METZENBERG 1996a).

Strain description. All *N. crassa* strains used in this study are described in Table 2 below. *Escherichia coli* K12 XL1-Blue MR (Stratagene) was the host for all bacterial manipulations.

TABLE 2 – Fungal strains used in this study

Name[a]	Genotype[b]	Origin
DLNCR63A	(*Sad-1*^{RIP}, *his-3*$^{+}$*::lpl*$^{\Delta}$(5192-6046)*::Sad-1*^{RIP}[301-3950]; *inl A*)	(LEE *et al.* 2003b)
DLNCR64A	(*Sad-1*^{RIP64}, *his-3*; *inl a*)	(LEE *et al.* 2003b)
DLNCR65A	(*his-3*; *Sms-3*^{RIP65}; *inl a*)	Progeny from RANCR43AΔ9 x (KYNCT02A + FGSC 4564)
DLNCR66A	(*Sad-1*^{RIP64}, *his-3*; *inl A*)	Progeny from DLNCR63A x (KYNCT02A + FGSC 4564)
DLNCR69A	(*Sad-1*^{RIP64}, *his-3*$^{+}$*::lpl*$^{\Delta}$ (5192-6046)*::Asm-1*$^{+}$[9336-3426]; *Asm-1*$^{\Delta}$ (3426-9336)*::hph*$^{+}$*::mcl-1*, *inl A*)	(LEE *et al.* 2003b)
DLNCR73A	(*Sad-1*^{RIP64}, *his-3*; *Sms-3*^{RIP65}; *inl A*)	Progeny from DLNCR66A x DLNCR65A
DLNCR74A	(*Sad-1*^{RIP64}, *his-3*; *Sms-3*^{RIP65}; *inl a*)	Progeny from DLNCR66A x DLNCR65A
DLNCR75A	(*his-3*; *dcl-2*^{RIP75}; *inl a*)	Progeny from RANCR44AΔ49 x DLNCR65A
DLNCR75B	(*his-3*; *dcl-2*^{RIP75}; *inl a*)	Progeny from RANCR05A x DLNCR75A
DLNCR76B	(*his-3*; *dcl-2*^{RIP75}; *Sms-3*^{RIP65}; *inl a*)	Progeny from DLNCR73A x DLNCR75A

TABLE 2 – Continued

Name[a]	Genotype[b]	Origin
DLNCR77A	(*his-3*; *dcl-2*^{RIP75}; *Sms-3*^{RIP65}; *inl A*)	Progeny from DLNCR73A x DLNCR75A
DLNCR80A	(*his-3*; *dcl-2*^{RIP75}; *inl A*)	Progeny from DLNCR73A x DLNCR75A
DLNCR88A	(*his-3*; *inl*; *Sms-2*^{RIP88} *a*)	(LEE *et al.* 2003b)
DLNCR93A	(*his-3*, *Rsp*^{RIP93}; *inl A*)	(PRATT *et al.* 2004)
DLNCR100A	(*his-3*; *inl*; *Sms-2*^{RIP88} *A*)	Progeny from DLNCR69A x DLNCR88A
DLNCR128A	(*his-3*$^{+}$*::lpl*$^{\Delta(5192\text{-}6046)}$*::hph*$^{+}$*::tk*$^{+}$; *Sms-3*^{RIP65}; *inl a*)	Progeny from DLNCT62A x DLNCR65A
DLNCT62A	(*his-3*$^{+}$*::lpl*$^{\Delta(5192\text{-}6046)}$*::hph*$^{+}$*::tk*$^{+}$; *inl A*)	(LEE *et al.* 2003a)
FGSC 4564	(*ad-3B cyh-1*^{R} *a*^{m1})	(GRIFFITHS and DELANGE 1978)
KBNCR05A	(*Rsp*^{RIP93}; *fl* A)	Progeny from DLNCR93A x RANCR50A
KBNCR06A	(*Rsp*^{RIP93}; *fl* a)	Progeny from DLNCR93A x RANCR50A
KYNCT02A	(*his-3*; *Asm-1*$^{\Delta(3426\text{-}9336)}$*::hph*$^{+}$*::mcl-1*, *inl a*)	(LEE *et al.* 2004)
KYNCT34A	(*his-3*$^{+}$*::lpl*$^{\Delta(5192\text{-}6046)}$*::Asm-1*$^{+}$[9336-3426]; *inl A*)	(LEE *et al.* 2004)
KYNCT35A	(*his-3*$^{+}$*::lpl*$^{\Delta(5192\text{-}6046)}$*::Asm-1*$^{+}$[9336-3426]; *inl a*)	(LEE *et al.* 2004)
MMNCR01A	(*fl*; *Sms-3*^{RIP65} *A*)	Progeny from RANCR49A x DLNCR65A
MMNCR08A	(*his-3*::*lpl*$^{\Delta(5192\text{-}6046)}$*::hph*$^{+}$*::tk*$^{+}$; *dcl-2*^{RIP75}; *inl A*)	Progeny from DLNCT62A x DLNCR75A
MMNCR09A	(*his-3*::*lpl*$^{\Delta(5192\text{-}6046)}$*::hph*$^{+}$*::tk*$^{+}$; *dcl-2*^{RIP75}; *Sms-3*^{RIP65}; *inl A*)	Progeny from MMNCR08A x MMNCT07A

TABLE 2 – Continued

Name[a]	Genotype[b]	Origin
MMNCR14A	(*his-3*; *Sms-3*^{RIP65}; *inl A*)	Progeny from MMNCR01A x RANCR06A
MMNCR15A	(*his-3*; *Sms-3*^{RIP65}; *inl*; *Sms-2*^{RIP88} *A*)	Progeny from DLNCR73A x DLNCR88A
MMNCR16A	(*his-3*; *Sms-3*^{RIP65}; *inl*; *Sms-2*^{RIP88} *a*)	Progeny from DLNCR73A x DLNCR88A
MMNCR19A	(*his-3*; *Sms-3*^{RIP19}; *inl A*)	Progeny from MMNCT18A x RANCR05A
MMNCR20A	(*his-3*; *Sms-3*^{RIP19}; *inl a*)	Progeny from MMNCR19A x DLNCR88A
MMNCR21A	(*his-3*; *Sms-3*^{RIP19}; *inl*; *Sms-2*^{RIP88} *A*)	Progeny from MMNCR19A x DLNCR88A
MMNCR22A	(*his-3*; *Sms-3*^{RIP19}; *inl*; *Sms-2*^{RIP88} *a*)	Progeny from MMNCR19A x DLNCR88A
MMNCT07A	(*his-3*$^{+}$*::lpl*$^{\Delta}$(5192-6046)*::Sms-3*[2542-(-767)]; *Sms-3*^{RIP65} *a*)	Transformation of DLNCR128A with pMMAM06[c]
MMNCT18A	(*his-3*$^{+}$*::lpl*$^{\Delta}$(5192-6046)*::Sms-3*[982-5850]; *Sms-3*^{RIP65}; *inl a*)	Transformation of DLNCR128A with pMMAM18[c]
RANCR05A	(*his-3; inl A*)	RANC Collection
RANCR06A	(*his-3; inl a*)	RANC Collection
RANCR49A	(*fl A*)	RANC Collection
RANCR50A	(*fl a*)	RANC Collection
RANCR43AΔ9	(*his-3*$^{+}$*::lpl*$^{\Delta}$(5192-6046)*::Sms-3*^{RIP}; *Sms-3*^{RIP}; *inl A*)	Progeny from RANCT43A x (KYNCT02A + FGSC4564)

TABLE 2 – Continued

Name[a]	Genotype[b]	Origin
RANCR44AΔ49	(*his-3*$^{+}$*::lpl*$^{\Delta(5192\text{-}6046)}$*::dcl-2*^{RIP}; *dcl-2*^{RIP}; *inl A*)	Progeny from RANCT44A x (KYNCT02A + FGSC4564)
RANCT43A	(*his-3*$^{+}$*::lpl*$^{\Delta(5192\text{-}6046)}$*::Sms-3*[1504-3449]; *inl A*)	Transformation of RANCR05A with pSMS-3[c]
RANCT44A	(*his-3*$^{+}$*::lpl*$^{\Delta(5192\text{-}6046)}$*::dcl-2*[PCR]; *inl A*)	Transformation of RANCR05A with pDCL-2[c]

[a]DLNC, FGSC, KBNC, KYNC, MMNC and RANC indicate strains constructed or provided for this study by Dong W. Lee, Fungal Genetics Stock Center, Kevin Baker, Kye-Yong Seong, Malcolm McLaughlin and Rodolfo Aramayo, respectively.

[b]Allele numbers or designations are: *A*, *mating type A*; *a*, *mating type a*; *adenine-3B*, *ad-3B* (12-17-114); a^{m1} is a mutant allele of the *a* idiomorph described in (GRIFFITHS and DELANGE 1978); *Ascospore maturation-1*, *Asm-1*; *cycloheximide resistant-1*, *cyh-1*^{R} (KH52r); *dicer-like-2*, *dcl-2* (RIP75); *fluffy*, *fl* (P); *histidine-3*, *his-3* (1-234-723); *hygromycin B phosphotransferase*, *hph*$^{+}$; *hygromycin B phosphotransferase* fused *in-frame* to the Herpes Simplex Virus *thymidine kinase* gene, *hph*$^{+}$*::tk*$^{+}$ (LUPTON *et al.* 1991); *inositol*, *inl* (89601); *lysophospholipase*, *lpl*$^{\Delta(5192\text{-}6046)}$ (see (LEE *et al.* 2004) for details); *myosin chain-like-1*, *mcl-1* (see (LEE *et al.* 2004) for details); *Roundspore*, *Rsp* (RIP93); *Suppressor of ascus dominance-1*, *Sad-1* (RIP64 and RIP66); *Suppressor of meiotic silencing-2*, *Sms-2* (RIP2 and RIP88); *Suppressor of meiotic silencing-3*, *Sms-3* (RIP19, RIP65 and RIP66).

[c]Described in Materials and Methods.

RIP-Mutagenesis. Alleles *Sms-3*^{RIP19} and *Sms-3*^{RIP65} were generated by integrating either a 4,869-bp (coordinates 982 to 5850--*Sms-3*^{RIP19}) or a 1,945-bp (coordinates 1504 to 3449--*Sms-3*^{RIP65}) *Sms-3* fragment at the *histidine-3* (*his*-3) chromosomal position, as described (ARAMAYO and METZENBERG 1996a; HAAG *et al.* 2003; LEE *et al.* 2003a). The duplication-bearing strains were then crossed to wild-type strains following standard procedures. Progeny without the duplication were screened and selected for isolates carrying *Sms-3*^{RIP}-alleles. These alleles were identified by extracting their DNA, digesting the DNA with both methylation-sensitive and methylation-insensitive restriction enzymes, fractionating the resulting products by electrophoresis on a 1% Agarose gel, transferring the DNA fragments to Nylon membranes, and probing the membranes with radio labeled primer-extended DNA fragments corresponding to the wild-type *Sms-3* region. A duplication-bearing strain (MMNCT07A) was generated by integrating a 3,309-bp fragment (coordinates -767 to 2542) of *Sms-3*, at the *his-3* locus, into a strain carrying the *Sms-3*^{RIP65} allele at its canonical chromosomal location. This MMNCT07A strain was then crossed to MMNCR08A. Progeny from this cross were processed as described above. The *dcl-2*^{RIP75} allele was generated following similar procedures. In this case RIP-mutagenesis was induced by a 2,402-bp *dcl-2* fragment corresponding to an internal segment of the coding region of the gene integrated at *his-3* locus.

DNA sequencing. Plasmid pMMAM19 (*Sms-3*^{RIP19} allele) was sequenced via the GeneJumper Primer Insertion Kit (Invitrogen) and the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (PEBiosystems, Foster City, CA, USA). We closed gaps in our sequence data by cloning a 5,617 bp fragment (coordinates 406 to 6022) from MMNCR19A into pBSK+ (Stratagene) with OMM012 and OMM013 (Table 1) as primers, and by sequencing unresolved regions with OMM016, OMM017, OMM018 and OMM019 (Table 1), using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and AmpliTaq DNA polymerase (PE Biosystems). Sequences were generated on an Applied Biosystems Model 377 automated DNA sequencer at GeneTechnologies Laboratory (Institute of Developmental and Molecular Biology, Texas A&M University, College Station, TX, USA).

Sequencing of *Sms*-3^{RIP19} reveals 389 GC-to-AT transition mutations uniformly spread across the duplicated region (Figure 3). The *Sms*-3^{RIP19} allele is 92% identical to *sms*-3^{+}. Only a fraction of the region corresponding to the *Sms*-3^{RIP65} and *dcl-2*^{RIP75} alleles were sequenced (Figure 3). The *Sms*-3^{RIP19} allele has a stop codon at position 1,234, and is thus predicted to encode a polypeptide 84 amino acid residues long. More than one premature stop codon was detected in sequenced regions of the *Sms*-3^{RIP65} and *dcl-2*^{RIP75} alleles. RIP mutations per Kbp of DNA were similar for all RIP alleles (data not shown).

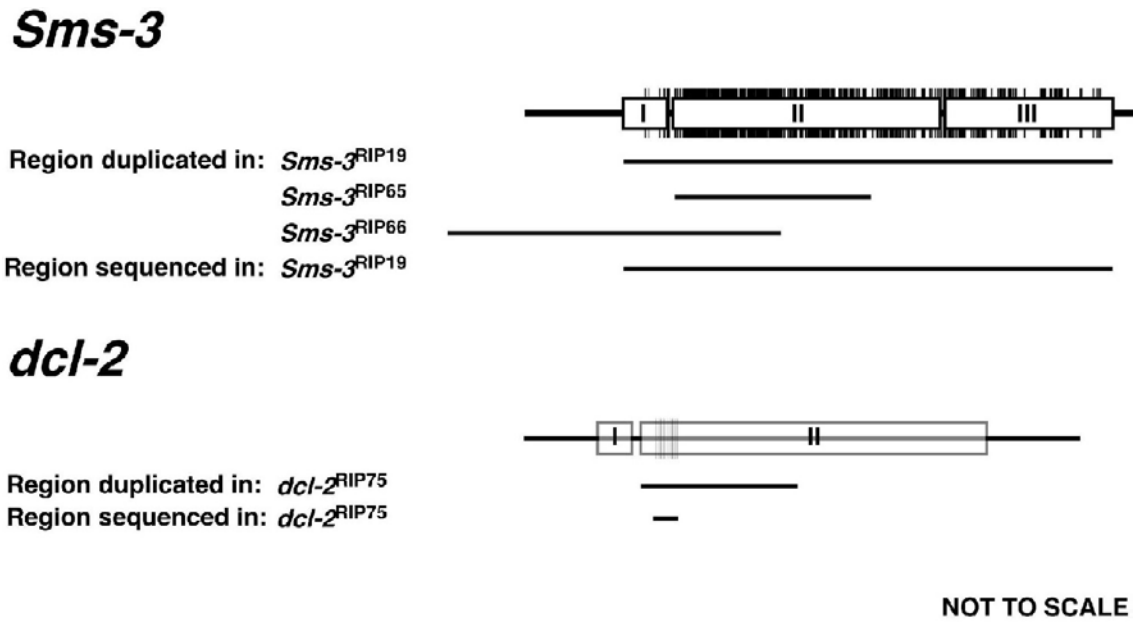


Figure 3 - Sequencing of the *Sms-3* and *dcl-2* RIP alleles. Several *Sms-3* and *dcl-2* RIP alleles were generated via the duplication and crossing techniques described in the Materials and Methods. *Sms-3*^{RIP19} and *dcl-2*^{RIP75} were sequenced within the RIP regions; the frequency of RIP mutation is displayed above. The relative percentages of mutations per Kb were similar across multiple alleles (data not shown).

Results

The Dicers of Neurospora. Sequencing of the *N. crassa* genome revealed the presence of two genes related to the Dicer proteins of other organisms (BORKOVICH *et al.* 2004; GALAGAN *et al.* 2003). Although originally named *dicer-like-1* (*dcl-1*, NCU08270.1) and *dicer like-2* (*dcl-2,* NCU06766.1) (BORKOVICH *et al.* 2004; GALAGAN *et al.* 2003), *dcl-1* was re-named *Suppressor of meiotic silencing-3* (*Sms-3*) based on its ability to suppress meiotic RNA silencing as induced by unpaired DNA (see below). The predicted proteins of these genes (SMS-3 and DCL-2, respectively) contain classical signatures associated with proteins of the Dicer family of ribonucleases. Both proteins carry a DEXDc (or ATP-binding site), a HELICc (or helicase and nucleotide binding region), a DUF283 (Domain of Unknown Function) and at least one RIBOc (RNA binding and Ribonuclease III activity) domain. The major difference between them is that SMS-3 contains two intact RIBOc domains, while DCL-2 contains one complete and one incomplete RIBOc domain (Figure 4). The predicted proteins of both SMS-3 and DCL-2 lack an obvious PAZ (Piwi/Argonaute/Zwille) domain (CERUTTI *et al.* 2000; LINGEL *et al.* 2003; SONG *et al.* 2003; TAHBAZ *et al.* 2004; YAN *et al.* 2003).

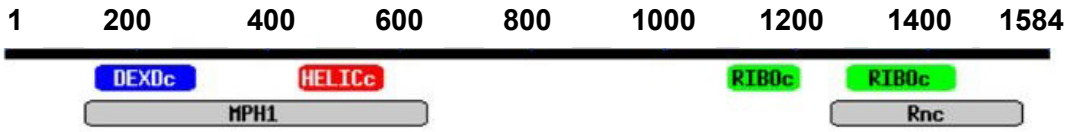
SMS-3 Dicer Protein

Organism: *Neurospora crassa*

Gene name: NCU08270.1

Gene identifier: GI_32416996

Predicted protein length: 528 amino acids



DCL-2 Dicer Protein

Organism: *Neurospora crassa*

Gene name: NCU06766.1

Gene identifier: GI_32413144

Predicted protein length: 507 amino acids

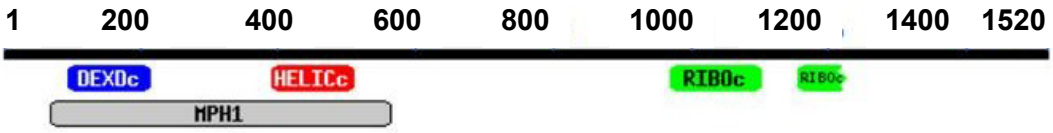


Figure 4 - Structural domains of the Dicers in *Neurospora crassa.* Each Dicer-like protein contains a single DEXDc domain (ATP-binding site) and a single HELICc (helicase) domain; however, SMS-3 contains two RIBOc (RNA binding and Ribonuclease III) domains, whereas DCL-2 contains only one RIBOc domain.

The Neurospora SMS-3 Dicer is required for sexual development. To investigate the functions of the Neurospora Dicers, we constructed strains carrying loss-of-function alleles in both *Sms-3* and *dcl-2* (*Sms-3*^{RIP65} and *dcl-2*^{RIP75}; see Materials and Methods and Table 2). To verify the loss-of-function of the *Sms-3* and *dcl-2* RIP'd alleles, we genetically combined the alleles and tested the ability of the resulting double-mutant strain of *Neurospora* to undergo vegetative RNA silencing (quelling). Given that the involvement of these Dicers in quelling has been previously established (CATALANOTTO *et al.* 2004), we expected the resulting strain (MMNCR09A) to be quelling-deficient. Silencing of the endogenous *albino-1* (*al-1*) gene involved in carotenoid biosynthesis was tested by integrating a construct capable of expressing a hairpin RNA homologous to the *al-1* transcript under the control of a repressible promoter at the *his-3* locus (ARAMAYO and METZENBERG 1996a; GOLDONI *et al.* 2004; HAAG *et al.* 2003; LEE *et al.* 2003a). Several transformants were selected, purified to homokaryosis as described in Lee (2003a), verified by Southern blot analysis and tested for silencing. In all cases, the resulting double-mutant strains tested were quelling-deficient (data not shown).

We next tested if strains carrying loss-of-function alleles in *Sms-3* or *dcl-2* presented developmental and/or metabolic abnormalities. For this, the ability of the resulting strains to complete the asexual and the sexual cycles was determined. Strains carrying the *dcl-2*^{RIP75} loss-of-function allele are

completely normal. We could not detect any major growth or developmental abnormalities. The strains conidiated normally, were female fertile and, in homozygous crosses, produced normal-looking perithecia that were indistinguishable from wild-type ones (see Figure 5, compare Panels A, and B with C; see also Table 3, crosses 1 and 2). These perithecia could mature and produce an abundant crop of ascospores that were mildly pale after being shot, but that developed into mature black ascospores over time (data not shown).

Similarly, strains carrying the *Sms-3*^{RIP19} and *Sms-3*^{RIP65} alleles also grew and conidiated normally. In addition, when these strains were inoculated onto nitrogen-limiting solid medium and incubated at room temperature, the formation of typical protoperithecial structures could be readily observed. Protoperithecia formed by these *Sms-3*^{RIP} mutants are functional based on the fact that when lawns of *Sms-3*^{RIP} mutants were fertilized with wild-type conidia of the opposite mating type, thousands of incipient perithecia per lawn were formed (data not shown). Surprisingly, however, despite their female fertility, and in contrast to what we observed with the *dcl-2*^{RIP} mutants, crosses homozygous for *Sms-3* loss-of-function alleles were severely underdeveloped and failed to produce any ascogenous tissue (see Figure 5, Panels I and J; see also Table 3, cross 8), thus precluding the possibility of ascospore development and maturation. The same was also true of the *Sad-1* and *Sms-2* RIP alleles (Figure 5, Panels E and G), confirming previous observations (LEE *et al.* 2003b; SHIU *et al.* 2001).

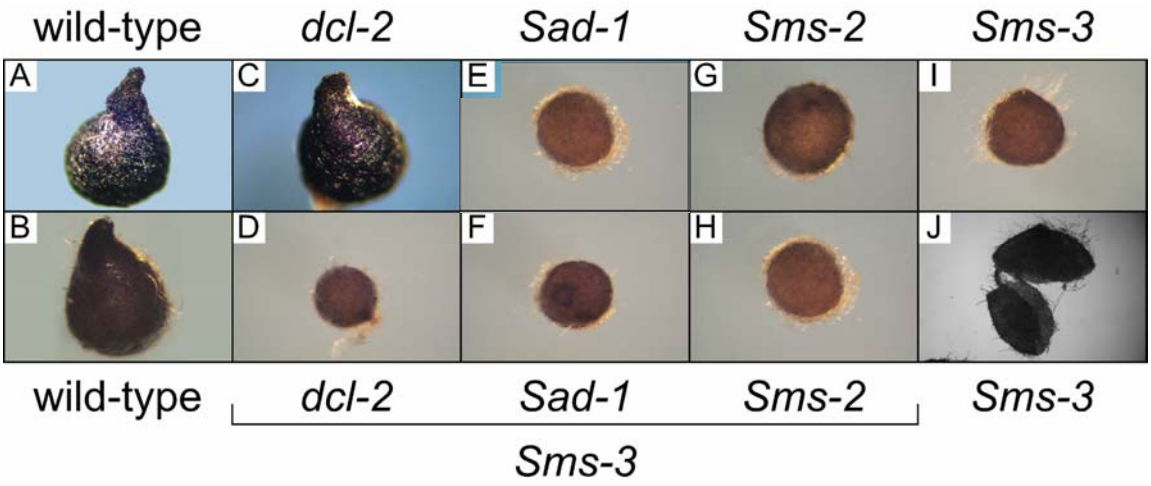


Figure 5 - Homozygous mutants in genes involved in meiotic silencing display severe sexual developmental defects. Perithecia were isolated, cleaned, and photographed using an Olympus SZ11 light microscope at ~60X magnification. Panels A through J depict representative sexual structures resulting from homozygous crosses for the following alleles: wild-type = A & B; *dcl-2* = C; *dcl-2 Sms-3* = D; *Sad-1* = E; *Sad-1 Sms-3* = F; *Sms-2* = G; *Sms-2 Sms-3* = H; and *Sms-3* = I & J. Note the formation of fully mature perithecia with a well-developed neck (Panels A, B, C and E) and the noticeably smaller perithecia with no or smaller necks formed by *Sms-2* and *Sms-3* mutants (Panels G and I, respectively), and by the corresponding double mutants (Panels D, F, and H). No internal ascogenous tissues were observed for strains carrying *Sms-3* loss-of-function alleles (Panel J). Table 3 depicts these results in tabular form.

TABLE 3 - SMS-3 is required for sexual development in *N. crassa*

	Relevant Genotype[a]						
		LGI	LGII	LGIV	LGVII		
Cross	*mat*	*Sad-1*	*dcl-2*	*Sms-3*	*Sms-2*	Parents	Results
1	*A*	*sad-1*$^+$;	*dcl-2*$^+$;	*sms-3*$^+$;	*sms-2*$^+$	RANCR05A	
	x					X	Fertile
	a	*sad-1*$^+$;	*dcl-2*$^+$;	*sms-3*$^+$;	*sms-2*$^+$	RANCR06A	

TABLE 3 - Continued

Cross	Relevant Genotype[a]					Parents	Results
		LGI	LGII	LGIV	LGVII		
	mat	*Sad-1*	*dcl-2*	*Sms-3*	*Sms-2*		
2	*A*	*sad-1*$^{+}$;	*dcl-2*^{RIP75};	*sms-3*$^{+}$;	*sms-2*$^{+}$	DLNCR80A	
	x					X	Fertile
	a	*sad-1*$^{+}$;	*dcl-2*^{RIP75};	*sms-3*$^{+}$;	*sms-2*$^{+}$	DLNCR75A	
3	*A*	*sad-1*$^{+}$;	*dcl-2*^{RIP75};	*Sms-3*^{RIP65};	*sms-2*$^{+}$	DLNCR77A	
	x					X	Barren
	a	*sad-1*$^{+}$;	*dcl-2*^{RIP75};	*Sms-3*^{RIP65};	*sms-2*$^{+}$	DLNCR76B	
4	*A*	*Sad-1*^{RIP64};	*dcl-2*$^{+}$;	*sms-3*$^{+}$;	*sms-2*$^{+}$	DLNCR66A	
	x					X	Barren
	a	*Sad-1*^{RIP64};	*dcl-2*$^{+}$;	*sms-3*$^{+}$;	*sms-2*$^{+}$	DLNCR64A	
5	*A*	*Sad-1*^{RIP64};	*dcl-2*$^{+}$;	*Sms-3*^{RIP65};	*sms-2*$^{+}$	DLNCR73A	
	x					X	Barren
	a	*Sad-1*^{RIP64};	*dcl-2*$^{+}$;	*Sms-3*^{RIP65};	*sms-2*$^{+}$	DLNCR74A	
6	*A*	*sad-1*$^{+}$;	*dcl-2*$^{+}$;	*sms-3*$^{+}$;	*Sms-2*^{RIP88}	DLNCR100A	
	x					X	Barren
	a	*sad-1*$^{+}$;	*dcl-2*$^{+}$;	*sms-3*$^{+}$;	*Sms-2*^{RIP88}	DLNCR88A	
7	*A*	*sad-1*$^{+}$;	*dcl-2*$^{+}$;	*Sms-3*^{RIP65};	*Sms-2*^{RIP88}	MMNCR15A	
	x					X	Barren
	a	*sad-1*$^{+}$;	*dcl-2*$^{+}$;	*Sms-3*^{RIP65};	*Sms-2*^{RIP88}	MMNCR16A	

TABLE 3 - Continued

Cross	Relevant Genotype[a]					Parents	Results
		LGI	LGII	LGIV	LGVII		
	mat	*Sad-1*	*dcl-2*	*Sms-3*	*Sms-2*		
8	*A*	*sad-1*$^+$;	*dcl-2*$^+$;	*Sms-3*^{RIP65};	*sms-2*$^+$	MMNCR14A	
	x					X	Barren
	a	*sad-1*$^+$;	*dcl-2*$^+$;	*Sms-3*^{RIP65};	*sms-2*$^+$	DLNCR65A	

[a]Complete genotypes are described in Table 2.

The sterility observed in *Sms-3*^{RIP} homozygous crosses is recessive, because heterozygous *Sms-3*^{RIP} x *sms-3*$^+$ crosses were fertile and produced viable ascospores (see below and data not shown). In addition, we tested if the sexual sterility of *Sms-3*^{RIP65} loss-of-function Dicer mutants could be complemented by the *sms-3*$^+$ *a*^{m1} ("helper") strain (FGSC 4564), whose nuclei contain a mutationally disabled mating type idiomorph that blocks them from participating in karyogamy (GRIFFITHS and DELANGE 1978; PERKINS 1984). For this, we constructed heterokaryons between the *sms-3*$^+$ *a*^{m1} strain and *Sms-3*^{RIP65} strains of either *A* (MMNCR14A) or *a* (DLNCR65A) mating types. When the resulting heterokaryons were crossed to each other they formed sterile perithecia that were indistinguishable from the ones formed by crosses between homokaryotic *Sms-3*^{RIP65} mutants (data not shown).

The barrenness of the *Sms-3*^{RIP} homozygous crosses resembles that observed for the *Sad-1* and *Sms-2* mutants (LEE *et al.* 2003b; SHIU *et al.* 2001, Figure 5). To test if all these mutants are blocked at the same developmental stage and to determine the epistatic relationship of *Sms-3* with *dcl-2*, *Sad-1*, and *Sms-2*, we first set up homozygous crosses for *dcl-2*^{RIP75}, *Sad-1*^{RIP64}, *Sms-2*^{RIP88}, and *Sms-3*^{RIP65} (Table 3, crosses 2, 4, 6, and 8). In parallel, homozygous crosses between *dcl-2*^{RIP75} *Sms-3*^{RIP65}, *Sad-1*^{RIP64} *Sms-3*^{RIP65}, and *Sms-2*^{RIP88} *Sms-3*^{RIP65} double mutants were also prepared (Table 3, crosses 3, 5, and 7). All crosses were set up in triplicate and were incubated at room temperature for 25 days, after which >20 individual perithecia from each cross were collected and analyzed. The results of these experiments are summarized in Figure 5 and Table 3. As expected, perithecia formed by the *dcl-2*^{RIP75} and *Sad-1*^{RIP64} mutants were indistinguishable from the ones produced by wild-type strains (Figure 5, compare Panels C and E with A and B). Surprisingly, we observed that the perithecia formed by *Sms-2*^{RIP88} and *Sms-3*^{RIP65} mutants were under-developed (Figure 5, compare Panels G and I with A and B). Interestingly, the developmental defects presented by crosses between *Sms-3*^{RIP65} mutants were consistently more severe than those observed in crosses between *Sms-2*^{RIP88} mutants (Figure 5, compare Panel I with G). *Sms-2*^{RIP88} perithecia, when opened, contained strands of largely underdeveloped ascogenous tissue (data not shown), whereas *Sms-3*^{RIP65} perithecia were completely devoid of

any ascogenous tissues (Figure 5, Panel J). Normal ascogenous tissue was readily observed in wild-type and *dcl-2*^{RIP75} perithecia (data not shown). Although *Sad-1*^{RIP64} perithecia contained some ascogenous tissue, we never observed any kind of spore development in these crosses (data not shown), consistent with what was observed before (SHIU *et al.* 2001). The sexual development behavior of *dcl-2*^{RIP75} *Sms-3*^{RIP65}, *Sad-1*^{RIP64} *Sms-3*^{RIP65}, and *Sms-2*^{RIP88} *Sms-3*^{RIP65} double mutants was particularly striking. In all cases, the presence of an *Sms-3*^{RIP65} loss-of-function allele was genetically epistatic to *dcl-2*^{RIP75}, *Sad-1*^{RIP64}, and *Sms-2*^{RIP88} (Figure 5, compare Panels D, F, and H with C, E, and G, respectively). These results clearly establish the requirement of at least a functional copy of the *sms-3*$^{+}$ Dicer gene in the early stages of the sexual developmental cycle of *Neurospora*. The discovery of a developmental role for Dicer is not without precedent; Dicer has been shown to process hairpin micro RNAs (miRNAs) that interact with RDE-1 homologues in *C. elegans*, suggesting a link between the RNAi machinery and developmental timing mechanisms (KETTING *et al.* 2001; GRISHOK *et al.* 2001).

The Neurospora SMS-3 Dicer is involved in meiotic silencing. The role of Dicer in RNA silencing has been well established (BARTEL 2004). To test the involvement of DCL-2 Dicer, we set up crosses homozygous for *dcl-2*^{RIP75} and induced meiotic silencing by unpairing a copy of either *Rsp* or *Asm-1.* In both

cases, silencing levels in the complete absence of DCL-2 Dicer were normal (data not shown). We therefore concluded that DCL-2 Dicer is completely dispensable for the induction and persistence of meiotic silencing.

In contrast to DCL-2, testing the involvement of SMS-3 Dicer is not simple. Crosses homozygous for *dcl-2*^{RIP75} yield perithecia with normal ascogenous tissues, whereas crosses homozygous for *Sms-3*^{RIP65} are barren. This barren state is also observed in crosses homozygous for *Sms-3*^{RIP19} (data not shown). The meiotic sterility observed in crosses involving homozygous loss-of-function *Sms-3* alleles complicates the conceptually simple experiment of testing meiotic silencing in the absence of *sms-3*$^{+}$. Given that heterozygous crosses are fertile (see above) and that meiotic silencing is reflexive (silencing of a silencer causes a loss of silencing) (LEE *et al.* 2003b; SHIU *et al.* 2001), we assayed silencing in heterozygous *sms-3*$^{+}$ x *Sms-3*^{RIP19} or *sms-3*$^{+}$ x *Sms-3*^{RIP65} crosses. In these crosses the inability of *sms-3*$^{+}$ to pair with *Sms-3*^{RIP19} or *Sms-3*^{RIP65} is predicted to induce partial silencing of the *sms-3*$^{+}$ allele itself (Figure 6, Panels A and B), as previously demonstrated for *Sad-1* and *Sms-2* (LEE *et al.* 2003b; PRATT *et al.* 2004; SHIU *et al.* 2001). This *cis*-silencing is also predicted to be dependent on the degree of heterology among the pairing alleles (PRATT *et al.* 2004); a higher degree of unpairing and silencing of the *sms-3*$^{+}$ allele is expected to occur in crosses involving highly homeologous (i.e., less

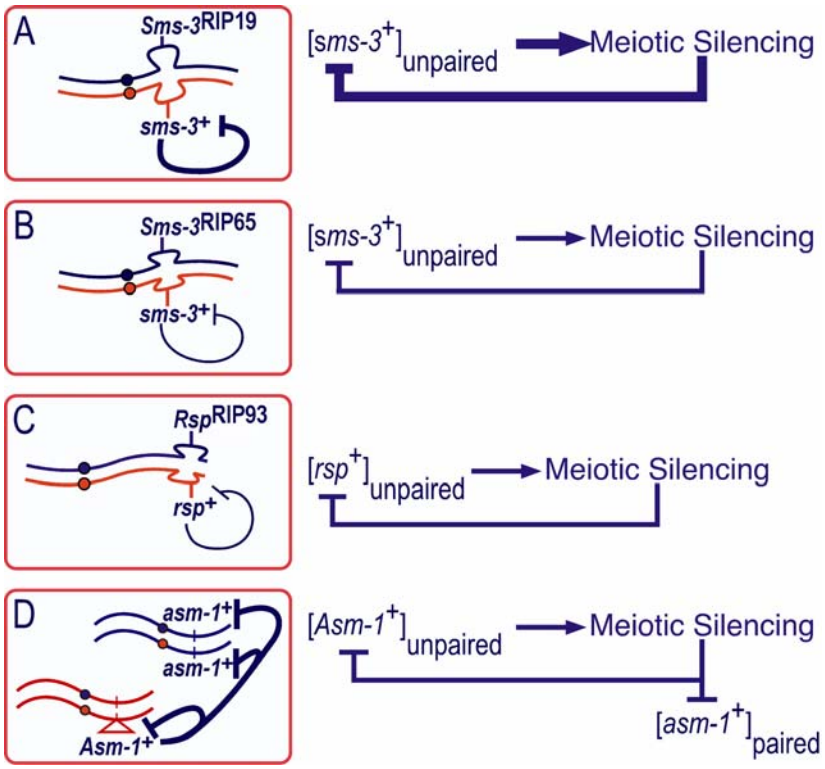


Figure 6 - Unpairing of *sms-3*$^{+}$ during meiosis induces silencing of the *sms-3*$^{+}$ allele itself. The diagrams represent diploid zygote cells corresponding to crosses involving different alleles of *Sms-3*, *Rsp*, and *Asm-1.* For simplicity, only one of the two sister chromatids is indicated, with circles representing centromeres. Relevant alleles and their relative position on chromosomes (*sms-3*$^{+}$, *Sms-3*^{RIP19}, and *Sms-3*^{RIP65} on LGIV; *rsp*$^{+}$, and *Rsp*^{RIP93} on LGI; and *asm-1*$^{+}$, and *Asm-1*$^{+}$ on LGV) are marked. The thickness of the lines with bars represents the predicted relative levels of silencing or unpairing (failure of *trans*-sensing) of different alleles. Dominance of the *Sms-3*^{RIP19} or *Sms-3*^{RIP65} alleles (Panels A and B) was assayed by determining the level of *cis*-silencing of *rsp*$^{+}$ in crosses between *rsp*$^{+}$ and *Rsp*^{RIP93} (Panel C), and the level of *trans*-silencing of *asm-1*$^{+}$ in crosses carrying the *Asm-1*$^{+}$ region inserted ectopically at the *his-3* locus. Because the unpaired *Asm-1*$^{+}$ region has no pairing partner on the homologous chromosome, it triggers silencing of all unpaired and paired copies of *Asm-1* present in the genome (Panel D). At the right of each diagram, a schematic representation of how the degree of unpairing of an allele directly correlates with the degree of induced silencing. In the case of silencers, the degree a silencer is silenced determines if it is possible to detect suppression of silencing (i.e., noise).

homologous; Figure 6, Panel A) alleles such as *Sms-3*^{RIP19} than in crosses involving less homeologous (i.e., more homologous; Figure 6, Panel B) alleles such as *Sms-3*^{RIP65} (PRATT *et al.* 2004). According to this model, *cis*-silencing of the *sms-3*$^{+}$ allele is expected to significantly decrease the amounts of *sms-3*$^{+}$ transcript and SMS-3-Dicer protein, to below that which is expected from a single fully functional gene. A reduction in the levels of SMS-3 Dicer protein should yield a reduction in silencing efficiency if *sms-3*$^{+}$ participates in meiotic RNA silencing. If there is enough SMS-3 protein in these heterozygous crosses (probably synthesized before the *sms-3*$^{+}$ gene is silenced) to allow meiosis to proceed, but not enough to maintain a fully functional meiotic silencing machinery, then we should be able to determine the involvement of *sms-3*$^{+}$ in meiotic RNA silencing by demonstrating a reduction in silencing efficiency in the presence of the *Sms-3*^{RIP19} and *Sms-3*^{RIP65} alleles (LEE *et al.* 2003b; PRATT *et al.* 2004; SHIU *et al.* 2001). Our experimental approach is conceptually simple: if *sms-3*$^{+}$ is required for meiotic RNA silencing, then the introduction of sms-3^{RIP} alleles to the system should reduce the efficiency of silencing.

We induced *cis*-meiotic silencing using the *Rsp*-reporter gene which, when unpaired, results in the production of round, viable ascospores. For this, strains carrying *rsp*$^{+}$ were crossed to strains carrying a highly mutated *Rsp*^{RIP93} allele

(PRATT *et al.* 2004) (Figure 6, Panel C). Silencing of the unpaired *rsp*$^{+}$ allele in the diploid cell was tested in an *sms-3*$^{+}$/*sms-3*$^{+}$ and in an *sms-3*$^{+}$/*Sms-3*^{RIP19} background. We first verified that crosses between fully wild-type strains produced over 98% mature, spindle-shaped ascospores (silencing negative control, Table 4, crosses 9 and 10). Next, we verified the presence of silencing in *rsp*$^{+}$/*Rsp*^{RIP93} heterozygous crosses, which produced only 5.5% and 5.3% spindle-shaped ascospores, depending on mating type (silencing positive control, Table 4, crosses 11 and 12) - similar to what was reported by PRATT *et al.* in 2004. This result shows that crosses homozygous for wild-type *sms-3*$^{+}$ efficiently silence *Rsp* in a heterozygous *rsp*$^{+}$/*Rsp*^{RIP93} cross. Significantly, the percentage of spindle-shaped ascospores observed in *rsp*$^{+}$/*Rsp*^{RIP93} *sms-3*$^{+}$/*Sms-3*^{RIP19} (experimental) crosses compared to the percentage observed in *rsp*$^{+}$/*Rsp*^{RIP93} *sms-3*$^{+}$/*sms-3*$^{+}$ (control) crosses was 84.0% and 78.5% vs. 5.3% and 5.5% (Table 4, crosses 11 through 14), depending on the mating type. Thus, in the presence of the *Sms-3*^{RIP19} allele silencing of *Rsp* was drastically reduced as indicated by the high percentage of spindle-shaped ascospores produced from the cross. The loss of a wild-type copy of *sms-3*$^{+}$ led to a drastic reduction in cis-silencing. These results clearly establish the participation of *Sms-3* in *cis*-meiotic RNA silencing.

TABLE 4 - Sms-3 is required for meiotic RNA silencing in *N. crassa*

	Relevant Genotype[b]									
		LGI			LGIV	LGVII				
Cross[a]	*mat*	*his-3*	*Rsp*		*Sms-3*	*Sms-2*	Parents	Spores Examined[c]	% Mature or Or Spindle Ascospores[c]	Observations	Suppress Silencing?
9	*A*	*his-3*	*rsp*$^+$;		*sms-3*$^+$;	*sms-2*$^+$	RANCR05A				
	x						x	Thousands	>98	Control	NA[d]
	a	*his-3*$^+$	*rsp*$^+$;		*sms-3*$^+$;	*sms-2*$^+$	RANCR50A				
10	*A*	*his-3*$^+$	*rsp*$^+$;		*sms-3*$^+$;	*sms-2*$^+$	RANCR49A				
	x						x	Thousands	>98	Control	NA[d]
	a	*his-3*	*rsp*$^+$;		*sms-3*$^+$;	*sms-2*$^+$	RANCR06A				
11	*A*	*his-3*	*rsp*$^+$;		*sms-3*$^+$;	*sms-2*$^+$	RANCR05A				
	x						x	10,285	5.3	Control[e]	No
	a	*his-3*$^+$	*Rsp*^{RIP93};		*sms-3*$^+$;	*sms-2*$^+$	KBNCR06A				
12	*A*	*his-3*$^+$	*Rsp*^{RIP93};		*sms-3*$^+$;	*sms-2*$^+$	KBNCR05A				
	x						x	10,385	5.5	Control[e]	No
	a	*his-3*	*rsp*$^+$;		*sms-3*$^+$;	*sms-2*$^+$	RANCR06A				

TABLE 4 - Continued

Cross[a]	Relevant Genotype[b]					Parents	Total Ascospores Examined[c]	% Mature or Or Spindle Ascospores[c]	Observations	Suppress Silencing?
	LGI			LGIV	LGVII					
	mat	*his-3*	*Rsp*	*Sms-3*	*Sms-2*					
13	*A*	*his-3*	*rsp*$^{+}$;	*Sms-3*^{RIP19};	*sms-2*$^{+}$	MMNCR19A				
	x					x	2,769	84.0	Experimental[e]	Yes
	a	*his-3*$^{+}$	*Rsp*^{RIP93};	*sms-3*$^{+}$;	*sms-2*$^{+}$	KBNCR06A				
14	*A*	*his-3*$^{+}$	*Rsp*^{RIP93};	*sms-3*$^{+}$;	*sms-2*$^{+}$	KBNCR05A				
	x					x	1,593	78.5	Experimental[e]	Yes
	a	*his-3*	*rsp*$^{+}$;	*Sms-3*^{RIP19};	*sms-2*$^{+}$	MMNCR20A				
15	*A*	*his-3*	*rsp*$^{+}$;	*sms-3*$^{+}$;	*Sms-2*^{RIP88}	DLNCR100A				
	x					x	1,414	87.6	Control[e]	Yes
	a	*his-3*$^{+}$	*Rsp*^{RIP93};	*sms-3*$^{+}$;	*sms-2*$^{+}$	KBNCR06A				
16	*A*	*his-3*$^{+}$	*Rsp*^{RIP93};	*sms-3*$^{+}$;	*sms-2*$^{+}$	KBNCR05A				
	x					x	1,604	88.1	Control[e]	Yes
	a	*his-3*	*rsp*$^{+}$;	*sms-3*$^{+}$;	*Sms-2*^{RIP88}	DLNCR88A				

TABLE 4 - Continued

Cross[a]	Relevant Genotype[b]					Parents	Total Ascospores Examined[c]	% Mature or Or Spindle Ascospores[c]	Observations	Suppress Silencing?
	LGI			LGIV	LGVII					
	mat	*his-3*	*Rsp*	*Sms-3*	*Sms-2*					
17	*A*	*his-3*	*rsp*$^{+}$;	*Sms-3*^{RIP19};	*Sms-2*^{RIP88}	MMNCR21A				
	x					x	1,508	97.5	Experimental[e]	Yes
	a	*his-3*$^{+}$	*Rsp*^{RIP93};	*sms-3*$^{+}$;	*sms-2*$^{+}$	KBNCR06A				
18	*A*	*his-3*$^{+}$	*Rsp*^{RIP93};	*sms-3*$^{+}$;	*sms-2*$^{+}$	KBNCR05A				
	x					x	2,008	96.0	Experimental[e]	Yes
	a	*his-3*	*rsp*$^{+}$;	*Sms-3*^{RIP19};	*Sms-2*^{RIP88}	MMNCR22A				
19	*A*	*his-3*	*rsp*$^{+}$;	*sms-3*$^{+}$;	*sms-2*$^{+}$	RANCR05A				
	x					x	21,152	0.7	Control[f]	No
	a	*his-3*$^{+}$*::Asm-1*$^{+}$[9336-3426]	*rsp*$^{+}$;	*sms-3*$^{+}$;	*sms-2*$^{+}$	KYNCT35A				
20	*A*	*his-3*$^{+}$*::Asm-1*$^{+}$[9336-3426]	*rsp*$^{+}$;	*sms-3*$^{+}$;	*sms-2*$^{+}$	KYNCT34A				
	x					x	12,098	1.8	Control[f]	No
	a	*his-3*	*rsp*$^{+}$;	*sms-3*$^{+}$;	*sms-2*$^{+}$	RANCR06A				

TABLE 4 - Continued

Cross[a]	Relevant Genotype[b] LGI *mat*	LGI *his-3*	LGI *Rsp*	LGIV *Sms-3*	LGVII *Sms-2*	Parents	Total Ascospores Examined[c]	% Mature or Or Spindle Ascospores[c]	Observations	Suppress Silencing?
21	*A*	*his-3*$^{+}$	*rsp*$^{+}$;	*Sms-3*^{RIP19}	*sms-2*$^{+}$	MMNCR19A				
	x					x	7,150	12.3	Experimental[f]	Yes
	a	*his-3*$^{+}$*::Asm-1*$^{+}$[9336-3426]	*rsp*$^{+}$;	*sms-3*$^{+}$;	*sms-2*$^{+}$	KYNCT35A				
22	*A*	*his-3*$^{+}$*::Asm-1*$^{+}$[9336-3426]	*rsp*$^{+}$;	*sms-3*$^{+}$;	*sms-2*$^{+}$	KYNCT34A				
	x					x	1,515	14.3	Experimental[f]	Yes
	a	*his-3*	*rsp*$^{+}$;	*Sms-3*^{RIP19};	*sms-2*$^{+}$	MMNCR20A				
23	*A*	*his-3*	*rsp*$^{+}$;	*sms-3*^{RIP65};	*sms-2*$^{+}$	MMNCR14A				
	x					x	832	3.5	Experimental[f]	No
	a	*his-3*$^{+}$*::Asm-1*$^{+}$[9336-3426]	*rsp*$^{+}$;	*sms-3*$^{+}$;	*sms-2*$^{+}$	KYNCT35A				
24	*A*	*his-3*$^{+}$*::Asm-1*$^{+}$[9336-3426]	*rsp*$^{+}$;	*sms-3*$^{+}$;	*sms-2*$^{+}$	KYNCT34A				
	x					x	7,394	3.5	Experimental[f]	No
	a	*his-3*	*rsp*$^{+}$;	*Sms-3*^{RIP65};	*sms-2*$^{+}$	DLNCR65A				

TABLE 4 - Continued

Cross[a]	Relevant Genotype[b]					Parents	Total Ascospores Examined[c]	% Mature or Or Spindle Ascospores[c]	Observations	Suppress Silencing?
	LGI			LGIV	LGVII					
	mat	*his-3*	*Rsp*	*Sms-3*	*Sms-2*					
25	*A*	*his-3*	*rsp*$^+$;	*sms-3*$^+$;	*Sms-2*^{RIP88}	DLNCR100A				
	x					x	1,095	42.0	Control[f]	Yes
	a	*his-3*$^+$::*Asm-1*$^+$[9336-3426]	*rsp*$^+$;	*sms-3*$^+$;	*sms-2*$^+$	KYNCT35A				
26	*A*	*his-3*$^+$::*Asm-1*$^+$[9336-3426]	*rsp*$^+$;	*sms-3*$^+$;	*sms-2*$^+$	KYNCT34A				
	x					x	1,185	41.3	Control[f]	Yes
	a	*his-3*	*rsp*$^+$;	*sms-3*$^+$;	*Sms-2*^{RIP88}	DLNCR88A				
27	*A*	*his-3*	*rsp*$^+$;	*Sms-3*^{RIP19};	*Sms-2*^{RIP88}	MMNCR21A				
	x					x	2,682	53.0	Experimental[f]	Yes
	a	*his-3*$^+$::*Asm-1*$^+$[9336-3426]	*rsp*$^+$;	*sms-3*$^+$;	*sms-2*$^+$	KYNCT35A				
28	*A*	*his-3*$^+$::*Asm-1*$^+$[9336-3426]	*rsp*$^+$;	*sms-3*$^+$;	*sms-2*$^+$	KYNCT34A				
	x					x	2,932	57.3	Experimental[f]	Yes
	a	*his-3*	*rsp*$^+$;	*Sms-3*^{RIP19};	*Sms-2*^{RIP88}	MMNCR22A				

TABLE 4 - Continued

Cross[a]	Relevant Genotype[b] LGI *mat*	LGI *his-3*	LGI *Rsp*	LGIV *Sms-3*	LGVII *Sms-2*	Parents	Total Ascospores Examined[c]	% Mature or Or Spindle Ascospores[c]	Observations	Suppress Silencing?
29	*A*	*his-3*	rsp^{+};	$Sms\text{-}3^{RIP65}$;	$Sms\text{-}2^{RIP88}$	MMNCR15A				
	x					x	18,001	53.8	Experimental[f]	Yes
	a	$his\text{-}3^{+}::Asm\text{-}1^{+}$[9336-3426]	rsp^{+};	$sms\text{-}3^{+}$;	$sms\text{-}2^{+}$	KYNCT35A				
30	*A*	$his\text{-}3^{+}::Asm\text{-}1^{+}$[9336-3426]	rsp^{+};	$sms\text{-}3^{+}$;	$sms\text{-}2^{+}$	KYNCT34A				
	x					x	18,519	53.6	Experimental[f]	Yes
	a	*his-3*	rsp^{+};	$Sms\text{-}3^{RIP65}$;	$Sms\text{-}2^{RIP88}$	MMNCR16A				

[a]When necessary, heterokaryons were constructed between the Griffiths sterile helper strain (FGSC 4564) and the required strains. On those occasions, sexual crosses were set up with both parents homokaryotic and also with both parents heterokaryotic. The results of the homokaryotic and of the heterokaryotic crosses were equivalent.

[b]Complete genotypes are described in Table 2.

[c]Genetic crosses were set up and scored as detailed in Materials and Methods.

[d]Not applicable (*i.e.,* meiotic silencing was not induced in these crosses).

[e]In these crosses, *cis*-meiotic silencing was induced by unpairing 4,391 bp of *Rsp* DNA (rsp^{+}[1961-6353]) at its canonical location in Linkage Group I. These crosses are not susceptible to RIP.

[f]In these crosses, *trans*-meiotic silencing was induced by unpairing 5,906 bp of *Asm-1* DNA ($Asm\text{-}1^{+}$[3430-9336]) at the *his-3* locus in Linkage Group I, in the presence of two paired copies of $asm\text{-}1^{+}$ in Linkage Group V. Due to the presence of the *Asm-1* duplicated region, these crosses are susceptible to RIP, which, if and when it occurs, reduces the frequency of black ascospores produced.

To demonstrate that *Sms-3* and *Sms-2* work in the same genetic pathway, we next tested the meiotic silencing behavior of single and double mutants, inducing *cis*-silencing as described above. In this case, the percentage of spindle-shaped ascospores produced in double mutant crosses vs. single mutant crosses was as follows: 97.5% and 96.0% for the *Sms-2*^{RIP88} *Sms-3*^{RIP19} double mutants, compared to 84.0% and 78.5% for *Sms-3*^{RIP19} and 87.6% and 88.1% for *Sms-2*^{RIP88} single mutants, depending on mating type (Table 4, crosses 13 through 18). These results are consistent with the idea that both *Sms-2* and *Sms-3* participate in meiotic silencing, since the disruption of both genes yields a greater loss of silencing efficiency than the disruption of either gene alone.

A similar logic was followed to test *trans*-meiotic silencing. Here, we induced silencing using the *Asm-1*-reporter gene which, when unpaired, results in the production of white and unviable ascospores. For this experiment, wild-type *asm-1*$^{+}$ strains carrying *Asm-1* DNA integrated at the *his-3* locus in Linkage Group I (LGI) (*his-3*$^{+}$*::Asm-1*; *asm-1*$^{+}$) were crossed to (*asm-1*$^{+}$) strains. Because the unpaired *Asm-1* region present in LGI (*his-3*$^{+}$*::Asm-1*) has no pairing partner on the homologous chromosome, it triggers meiotic silencing, which in turn silences all unpaired and paired homologous copies present in the genome (LEE *et al.* 2004; SHIU *et al.* 2001). We first demonstrated that crosses between *sms-3*$^{+}$ wild-type strains produced over 98% mature, black ascospores

in the absence of unpaired *Asm-1* DNA (silencing negative control, Table 4, crosses 9 and 10). Next, we verified the presence of silencing in crosses containing unpaired *Asm-1* DNA, which produced only 0.7% and 1.8% viable black ascospores, depending on mating type (silencing positive control, Table 4, crosses 19 and 20) - identical to the level previously reported for *Asm-1* by LEE *et al.* in 2004. The percentage of black, mature ascospores observed in *sms-3*$^{+}$/*Sms-3*^{RIP19} (experimental) crosses vs. that observed in *sms-3*$^{+}$/*sms-3*$^{+}$ (control) crosses was: 12.3% and 14.3% vs. 0.7% and 1.8%, depending on mating type (Table 4, crosses 19 through 21). Just as with cis-silencing, the presence of partially unpaired *Sms-3* led directly to a reduction in trans-silencing efficiency. These results are consistent with those obtained previously, and implicate *Sms-3* in *trans*-meiotic RNA silencing.

If the degree of unpairing of *sms-3*$^{+}$ is dependent on its level of homology to *Sms-3*^{RIP} alleles, as was observed for the interaction of *rsp*$^{+}$ with *Rsp*^{RIP} alleles (PRATT *et al.* 2004), and if *sms-3*$^{+}$ is involved in meiotic silencing, then silencing should be weaker in heterozygous *sms-3*$^{+}$/*Sms-3*^{RIP19} than in *sms-3*$^{+}$/*Sms-3*^{RIP65} crosses. This is because the region mutated in the *Sms-3*^{RIP65} allele is smaller than the one in the *Sms-3*^{RIP19} allele (Figure 3), and should result in a less unpaired *sms-3*$^{+}$ allele, which should in turn yield more efficient meiotic silencing. This prediction was confirmed. The percentage of black,

mature ascospores produced by these experimental and control crosses was as follows: 12.3% and 14.3% for the *sms-3*$^{+}$/*Sms-3*^{RIP19} (large region of mutation) crosses, 3.5% and 3.5% for the *sms-3*$^{+}$/*Sms-3*^{RIP65} (small region of mutation) crosses, and 0.7% and 1.8% for *sms-3*$^{+}$/*sms-3*$^{+}$ (control) crosses, depending on mating type (Table 4, crosses 19 through 24). This confirms the semi-dominant and semi-recessive nature of the loss-of-function *Sms-3*^{RIP19} and *Sms-3*^{RIP65} alleles, because the level of suppression of silencing increased as the severity of mutation to *Sms-3* increased.

To determine the interaction of the *Sms-3* Dicer and the *Sms-2* Argonaute genes in *trans*-silencing, we induced silencing as described above, and tested the meiotic silencing behavior of the single and double mutants. The percentage of black, mature ascospores produced by the double mutant (experimental) crosses as compared to the percentage produced by single mutant (control) crosses was as follows: 53.0% and 57.3% for *Sms-2*^{RIP88} *Sms-3*^{RIP19} and 53.8% and 53.6% for *Sms-2*^{RIP88} *Sms-3*^{RIP65} (depending on mating type), as compared to 12.3% and 14.3% for *Sms-3*^{RIP19}, 3.5% and 3.5% for *Sms-3*^{RIP65}, and 42.0% and 41.3% for *Sms-2*^{RIP88} (depending on mating type, Table 4, crosses 21 through 30). These results confirm that both *Sms-2* and *Sms-3* are required for meiotic silencing, because the level of suppression of silencing is higher in the double mutant crosses than in any single mutant cross.

Discussion

In this work we constructed and studied strains of *N. crassa* carrying mutations in *sms-3*$^{+}$ and *dcl-2*$^{+}$, the two Dicer ribonuclease genes encoded for in the *Neurospora* genome. We found that *dcl-2*$^{+}$ is completely dispensable for normal sexual development, as well as for meiotic RNA silencing. In contrast, *sms-3*$^{+}$ is required for both sexual development and meiotic RNA silencing. These results directly implicate the action of a Dicer ribonuclease in meiotic RNA silencing, and lend further support to the idea that meiotic RNA silencing is an RNA interference-related phenomenon.

Most Dicer ribonucleases described to date play central roles in RNA silencing. This is consistent with the idea that this group of enzymes evolved as part of an ancient genome defense mechanism designed to preserve genome integrity (ANANTHARAMAN *et al.* 2002; ARAVIND *et al.* 2000). In this scenario, the presence and accumulation of dsRNA probably signaled cells of an imminent invasion by mobile DNA or RNA elements, whose activity could be easily controlled by a mechanism akin to RNA interference. This, in turn, resulted in the swift and complete destruction of all messages with homology to the initial dsRNA trigger signal. Over evolutionary time, the efficiency of this genome-defense pathway likely improved, and variations of the pathway were likely recruited to control development. A living relic of these evolutionary forces is reflected in the

variable number of Dicer ribonucleases encoded by different genomes (MEISTER and TUSCHL 2004). They range from one member in *Homo sapiens* and *C. elegans*; two in *D. melanogaster*, *N. crassa* and *Magnaporthe oryzae*; and four in *A. thaliana.* Obviously, developmental complexity and the copy number of genes coding for Dicer proteins do not correlate, which indirectly hints at the flexibility of function and potential complexity of the pathways that are involved in these different organisms.

The involvement of small RNAs in vastly different, yet interconnected biological phenomena has placed RNA and the dsRNA-specific RNase-III-type Dicer endonucleases at the center stage of biology (BARTEL 2004; MEISTER and TUSCHL 2004). In the last few years, small RNAs have been demonstrated to be involved in mRNA degradation, translational inhibition, chromatin modification, and genome re-arrangement (AMBROS 2004; BAULCOMBE 2004; HANNON and ROSSI 2004; LIPPMAN and MARTIENSSEN 2004; MATZKE and BIRCHLER 2005; MEISTER and TUSCHL 2004; MELLO and CONTE 2004; MOCHIZUKI and GOROVSKY 2005). In plants and animals, RNase-III-type endonucleases generate at least two classes of small non-coding RNAs, siRNAs and miRNAs. *D. melanogaster* is an appropriate case study. Drosophila has three RNase-III-type endonucleases: Drosha, which is required for the processing of pri-miRNA into pre-miRNA--but cannot act on long-dsRNA; and two paralogues, Dicer-1, which preferentially cleaves pre-miRNA into miRNA and Dicer-2, which is required for processing of

long-dsRNA into siRNAs. In *Neurospora*, CATALANOTTO *et a*l. (2002) showed that both DCL-1 and DCL-2 are sufficient for the vegetative quelling pathway, and thus redundant. In contrast to the redundancy of Dicer function observed during vegetative development, *dcl-2* has no apparent in the meiotic silencing pathway of *Neurospora*, while *sms-3* is essential.

The current model for the meiotic silencing pathway of *N. crassa* (Figure 7) invokes two main stages: *trans*-sensing and meiotic RNA silencing. The *trans*-sensing step is complex. It involves active sensing and evaluation of homologous chromosomes in meiosis. During this process, paired regions do not affect normal development, but unpaired regions do so by activating the silencing pathway. We know that both unpaired and homeologous DNA regions can activate meiotic silencing. It remains to be determined, however, if these two different insults are being recognized by the same molecular machinery, or by separate apparatuses impinging on meiotic silencing. Extensive study (KUTIL *et al.* 2003; LEE *et al.* 2003b; LEE *et al.* 2004; PRATT *et al.* 2004) revealed a set of non-mutually exclusive properties required for meiotic silencing: (1) The larger the loop of unpaired DNA, the better the silencing; (2) the higher the percentage

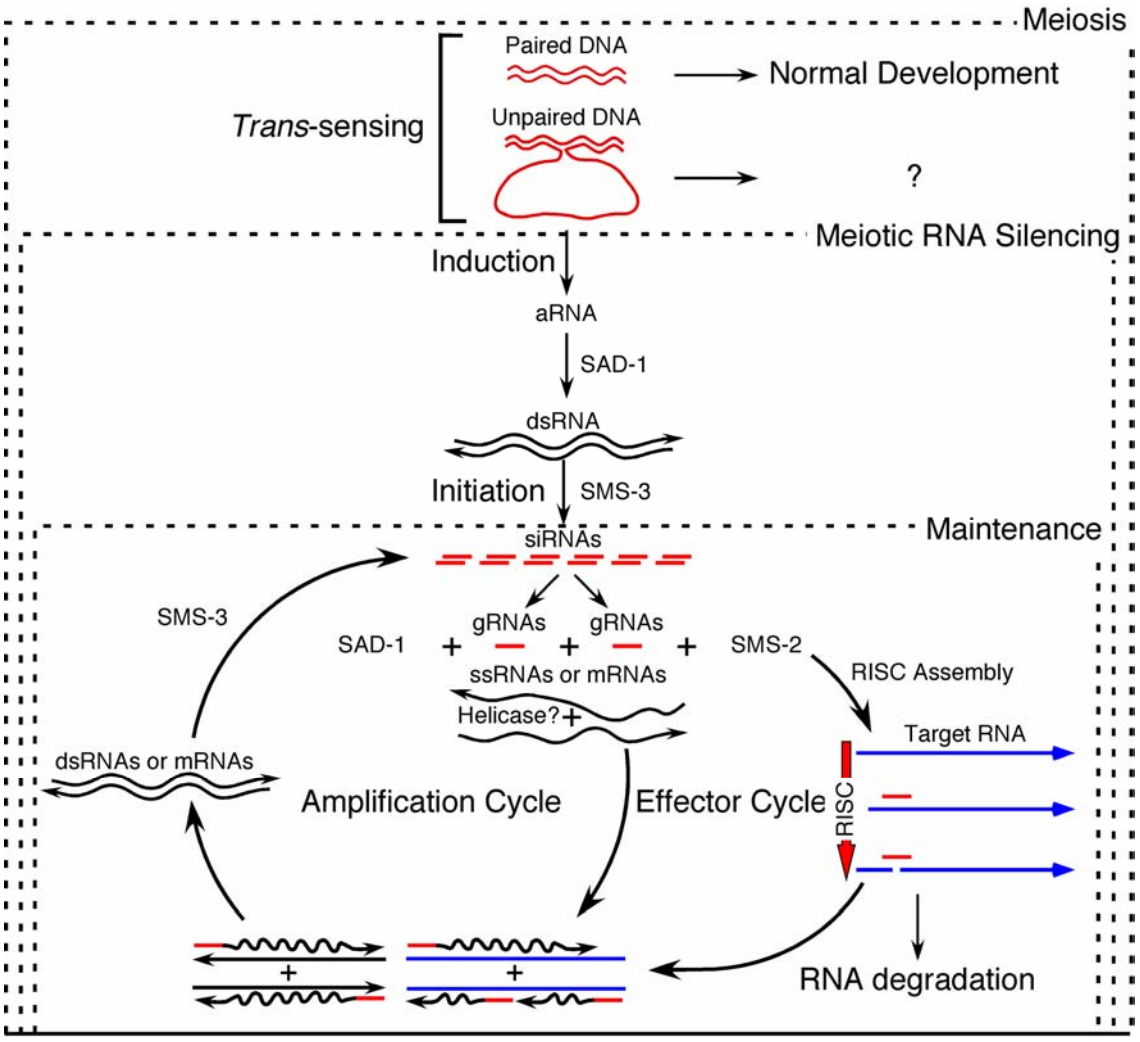


Figure 7 - A model for meiotic RNA silencing in *Neurospora crassa*. This figure shows both the proposed *trans*-sensing and meiotic RNA silencing stages, and the steps that would define the induction, initiation, and maintenance stages of meiotic silencing. The relationships between the amplification and effector cycles and other pathways predicted to play a role in the process (e.g., RNA degradation) are indicated.

of homology the unpaired region has to the reporter transcript, the better the silencing; (3) the silencing signal produced by an unpaired loop does not extend to paired regions immediately adjacent to the loop; (4) for a gene to be silenced in *cis* or in *trans*, its canonical promoter need not be present in the loop of

unpaired DNA; (5) unpairing does not affect the ability of a gene promoter to direct transcription at a later developmental time; and (6) DNA methylation, directly or indirectly, affects meiotic *trans*-sensing, but not meiotic silencing. The *trans*-sensing step is known to occur early in meiosis, during diploid development (ARAMAYO and METZENBERG 1996b). Although it is tempting to hypothesize that meiotic *trans*-sensing occurs during pachytene, when homologous chromosomes are the most intimate, it is currently unknown whether chromosome synapsis is required for *trans*-sensing, or if *trans*-sensing occurs at an earlier stage, perhaps during chromosome alignment. Further, it is also currently unknown if double-stranded breaks (DSB) play a role in the process and if they do, at which stage.

According to our model, a failure in *trans*-sensing triggers the start of meiotic RNA silencing, a process composed of: induction, initiation, maintenance, and resetting. The induction step involves the synthesis of aberrant RNA (aRNA) into dsRNA by an RNA-dependent RNA polymerase (RdRP). Given that silencing can be very efficient, even if truncated versions of a reporter gene are present in the loop of unpaired DNA (LEE *et al.* 2004), it is likely that aRNAs neither carry nor need poly(A) tails to participate fully in meiotic silencing. It is likely that these "truncated" transcripts must either be immediately converted into dsRNAs by the SAD-1-RdRP or somehow escape the RNA-quality-control mechanisms active in

eukaryotic cells (MOORE 2002), assuming that those mechanisms are functional during early meiosis.

Our model next suggests that the presence of dsRNA triggers the initiation step, which involves the conversion of the dsRNA trigger into siRNAs. This step was predicted to be Dicer-dependent (LEE *et al.* 2003b). Our work here confirms this early prediction and suggests that this step is in fact SMS-3-dependent. It also suggests that the production of siRNAs by the action of SMS-3 on dsRNA template likely generates the nucleus-restricted diffusible signal required for *trans*-silencing.

The maintenance of meiotic silencing would next require the amplification and effector cycles. The use of guide-single-stranded RNAs (gRNAs) as primers, and single-stranded RNA (ssRNA) as template by SAD-1-RdRP generates fresh dsRNA template for SMS-3. In parallel, the gRNAs generated by both the initiation step and the amplification cycles are likely to be incorporated into a RNA Induced Silencing Complex (RISC), of which the Argonaute-like protein SMS-2 is predicted to be the engine directing endonucleolytic cleavage of mRNA or ssRNA (LEE *et al.* 2003b). As discussed in Chapter I, RISC has been identified in *Drosophila* as a Dicer/dsRBP heterodimer which recruits Argonaute to form a holo-complex (PHAM, J.W. and E.J. SONTHEIMER, 2005). The final step, resetting, likely occurs late in spore development or in early germination.

Finally, it is important to emphasize that although the blueprint of the meiotic RNA silencing pathway closely resembles the RNA silencing pathways of other organisms, it is likely that meiotic silencing is a nuclear-specific pathway (MATZKE and BIRCHLER 2005). As such, the detailed characterization of the main players of this pathway and that of their interactions will surely be informative.

CHAPTER III

CONCLUSIONS AND FUTURE DIRECTIONS

The conclusions drawn from this work are important step in understanding the meiotic silencing phenomenon, but several questions remain. It is appropriate both to summarize the most relevant findings of this work and to address a few lingering questions. The single most important conclusion to be drawn from my study of *Sms-3* is that strong evidence exists to support our hypothesis that meiotic silencing is an RNA-mediated phenomenon. Several years ago, we began a set of experiments based on an attractive yet unsupported hypothesis that *Neurospora crassa* regulates its genome through the application of an RNA-induced silencing mechanism. This hypothesis had its roots in the discovery that the presence of unpaired regions of DNA during meiosis leads to a failure in meiotic transvection ("*trans*-sensing") in that region, and then to a subsequent silencing of all copies of any gene found in the unpaired region - even if a functional copy of that gene is found in another region of the genome (ARAMAYO and METZENBERG, 1996; ARAMAYO *et al.*, 1996).

Although the discovery of these associated phenomena was intriguing, a convincing explanation for the observed events was at first elusive. The discovery of the *Sad-1* mutant and its attenuating effect on meiotic silencing (SHIU *et al.*, 2001), however, clearly raised a question as to whether meiotic

silencing is in fact an RNA-mediated mechanism. As noted earlier, *Sad-1* is an RNA-dependent RNA Polymerase (RdRP). The involvement of an RdRP in the meiotic silencing mechanism of *N. crassa*, when examined with the knowledge that RdRPs are an integral component of RNA-mediated silencing in other organisms, demanded a careful examination of whether additional components of RNA-mediated silencing could be detected in the meiotic cycle of *N. crassa*. It was at this time that our laboratory proposed a model for RNA-mediated meiotic silencing in *N. crassa* that includes an RdRP, an Argonaute-like protein, and a Dicer-like protein, all functioning in a mechanism closely analogous to the RISC complex identified in *Drosophila* and suggested for other model organisms. The discovery of the activity of an Argonaute-like protein encoded for by *Sms-2* in meiotic silencing (LEE *et al.*, 2003b) strengthened our hypothesis that meiotic silencing is an RNA-mediated phenomenon. At this point, two of the three most commonly observed components of RNA-mediated silencing were identified in the meiotic cycle of *N. crassa*. Of course, the efficacy of our model is supported further by the previous discovery of an RdRP, Argonaute-like protein, Rec-Q DNA helicase, and two redundant Dicer-like proteins in the quelling pathway of *N. crassa* (CATALONOTTO *et al.*, 2004; CATALONOTTO *et al.*, 2002; COGONI and MACINO, 1999a). It thus seemed inevitable that a Dicer-like protein would also be involved in the meiotic silencing pathway of *N. crassa*.

The focus of this work thus became the identification of a Dicer-like activity in the meiotic silencing pathway of *N. crassa*. Through the application of basic genetic and molecular biology techniques, we have successfully demonstrated the involvement of a Dicer-like protein in the meiotic silencing pathway that is encoded for by a gene we have named *Sms-3*. The experimental data clearly indicate that the disruption of *Sms-3* leads to a detectable decrease in silencing efficiency, as indicated by the increased activity of reporter genes that would have otherwise been silenced by the presence of unpaired copies of the reporter genes. The identification of a Dicer-like protein in the meiotic silencing pathway significantly strengthens our model of an RNA-mediated mechanism due to the very nature of Dicer activity (RNA cleavage); it can thus be concluded that a Dicer-like protein acts in meiotic silencing, and strongly suggested that such a mechanism must be RNA-mediated.

Although a Dicer-like protein has been identified in support of our silencing model, the precise nature of the protein's activity cannot be elucidated from our findings at this time. For example, it could be reasonably predicted that the activity of a Dicer-like protein would yield detectable levels of siRNAs in meiotic cells. However, experiments in our laboratory previously failed to detect any such siRNAs. This may be due to a scarcity or localization of siRNAs in meiotic tissues. This failure to identify the presence of siRNAs or a distinct transitive RNAi process in N. crassa does not, however, alter the fact that a Dicer-like

protein plays a significant role in meiotic silencing. It remains a subject of further study to identify the precise nature of Dicer's role in the mechanism.

Further study of *Sms-3* and the phenomenon of meiotic silencing could be pursued through the application of biochemistry, cytogenetic, and traditional genetic techniques. Biochemical methods are difficult to apply in *N. crassa*, however, because it is essentially impossible to purify diploid-specific cells since meioses among the cells are not synchronized. It will thus be of greater value to pursue the study of *Sms-3* and meiotic silencing through the application of genetic traditional and novel cytogenetic techniques. The application of traditional genetic techniques to study meiotic silencing in *N. crassa* has been developed and streamlined in our laboratory, and we are currently applying large-scale suppression analyses to identify a number of novel mutant alleles that have a range of effects on silencing (data not shown). Our standard suppression analysis consists of subjecting conidia to an ultraviolet-induced DNA insult, crossing the conidia to strains containing the *Asm-1* and *Rsp* reporter genes, and screeing for crosses that demonstrate some degree of suppression of meiotic silencing. These suppression analyses are likely to yield a wealth of information and new players in the pathway and have proven to be a valuable tool in the past, having been used to identify the *Sad-1* mutant allele that first hinted at a connection between meiotic transvection and RNA-mediated silencing. It might also prove helpful to develop novel cytogenetic approaches to

study the activity of meiotic silencing in meiotically active cells, such as the ability to view meiotic cells in the microscope (perhaps via a fluorescent tag) under conditions conducive to silencing.

The study of meiotic silencing in *N. crassa* should prove to be significant for a number of reasons. It is of great interest to fungal biologists to fully understand the mechanisms of silencing, since this process can have such a profound effect on the genome and development of the organism. Interest In this phenomenon is further fueled by the realization that the meiotic silencing mechanism employed by *N. crassa* utilizes components that are remarkably similar to those seen in a variety of other model organisms. In this respect, studies in *N. crassa* will contribute to a better understanding of a highly conserved method of genetic defense. However, there is even greater significance to be found in the fact that the study of meiotic silencing may provide invaluable insight into developmental processes seen in a variety of organisms, including mammals. It has recently been shown that a form of meiotic silencing occurs in the mammalian organism *Mus musculus* (BAARENDS *et al.*, 2005; TURNER *et al.*, 2005). Specifically, it has been shown that unsynapsed (unpaired) chromosomes are silenced in mouse cells (male and female) during meiosis in a mechanism that involves the tumor suppressor BRCA1 (TURNER *et al.*, 2005). Turner *et al.* specifically note the commonality of meiotic silencing between *M. musculus* and *N. crassa* (TURNER *et al.*, 2005). The discovery of a meiotic silencing phenomenon in mammals

underscores the importance of establishing a fuller understanding of the system in all organisms in which it occurs, including *Neurospora*.

A fuller understanding of meiotic silencing may also be informative to the study of other pairing-related phenomena. Included among these are: non-disjunction events which, due to a failure of chromosome pairing, cause developmental abnormalities such as spontaneous abortion and Down's Syndrome (HASSOLD and HUNT 2001); imprinting, which is essential for normal development and which is dependent upon proper chromosomal pairing (CONSTANCIA *et al.*, 1998; HALL *et al.*, 1997; LALANDE 1996; LASALLE and LALANDE 1996; RIESSELMANN and HAAF 1999); pairing-dependent phenomena that influence gene expression, as seen in *Drosophila* (HENIKOFF and COMAI 1998; PIRROTTA 1999; WU and MORISS 1999) and plants (MATZKE *et al.*, 2001); and undoubtedly many others. In short, the future study of meiotic silencing in *N. crassa* may yield discoveries with implications that reach far beyond the arena fungal sexual development.

REFERENCES

AMBROS, V., 2004 The functions of animal microRNAs. Nature **431**: 350-355.

ANANTHARAMAN, V., E. V. KOONIN and L. ARAVIND, 2002 Comparative genomics and evolution of proteins involved in RNA metabolism. Nucleic Acids Research **30**: 1427-1464.

ARAMAYO, R., and R. L. METZENBERG, 1996a Gene replacements at the *his-3* locus of *Neurospora crassa*. Fungal Genetics Newsletter **43**: 9-13.

ARAMAYO, R., and R. L. METZENBERG, 1996b Meiotic transvection in fungi. Cell **86**: 103-113.

ARAMAYO, R., Y. PELEG, R. ADDISON and R. METZENBERG, 1996 *Asm-1*$^{+}$, a *Neurospora crassa* gene related to transcriptional regulators of fungal development. Genetics **144**: 991-1003.

ARAVIND, L., H. WATANABE, D. J. LIPMAN and E. V. KOONIN, 2000 Lineage-specific loss and divergence of functionally linked genes in eukaryotes. Proceedings of the National Academy of Sciences USA **97**: 11319-11324.

ARAVIND, L., and E.V. KOONIN, 2001 A natural classification of ribonucleases, Methods of Enzymology **341**: 3-28.

AUSUBEL, F. M., R. BRENT, R. E. KINGSTON, D. D. MOORE, J. A. SMITH *et al.* (Editors), 1987 *Current Protocols in Molecular Biology*. John Wiley and Sons, New York.

BAARENDS, W. M., E. WASSENAAR, R. VAN DER LAAN, J. HOOGERBRUGGE, E. SLEDDENS-LINKELS *et al.*, 2005 Silencing of unpaired chromatin and histone H2A ubiquitination in mammalian meiosis. Molecular and Cellular Biology **25**: 1041-1053.

BARTEL, D. P., 2004 MicroRNAs: genomics, biogenesis, mechanism, and function. Cell **116**: 281-297.

BAULCOMBE, D., 2004 RNA silencing in plants. Nature **431**: 356-363.

BERNSTEIN, E., A.A. CAUDY, S.M. HAMMOND and G.J. HANNON, 2001 Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409**: 295-296.

BERNSTEIN, E., S.Y. KIM, M.A. CARMELL, E.P. MURCHISON, H. ALCORN *et al.*, 2003 Dicer is essential for mouse development. Nature Genetics **35**: 215-217.

BORKOVICH, K. A., L. A. ALEX, O. YARDEN, M. FREITAG, G. E. TURNER *et al.*, 2004 Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism. Microbiology and Molecular Biology Reviews **68**: 1-108.

BOUTET, S., F. VAZQUEZ, J. LIU, C. BECLIN, M. FAGARD, *et al.*, 2003 *Arabidopsis HEN1*: A genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and visurs resistance. Current Biology **13**: 843-848.

CARMELL, M.A., Z. XUAN, M.Q. ZHANG and G.J. HANNON, 2002 The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes and Development **16**: 2733-42

CATALANOTTO, C., G. AZZALIN, G. MACINO and C. COGONI, 2000 Gene silencing in worms and fungi. Nature **404**: 245.

CATALANOTTO, C., G. AZZALIN, G. MACINO and C. COGONI, 2002 Involvement of small RNAs and role of the *qde* genes in the gene silencing pathway in Neurospora. Genes and Development **16**: 790-795.

CATALANOTTO, C., M. PALLOTTA, P. REFALO, M. S. SACHS, L. VAYSSIE *et al.*, 2004 Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in *Neurospora crassa*. Molecular and Cellular Biology **24**: 2536-2545.

CERUTTI, L., N. MIAN and A. BATEMAN, 2000 Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. Trends in Biochemical Sciences **25**: 481-482.

CHICAS, A., C. COGONI and G. MACINO, 2004 RNAi-dependent and RNAi-independent mechanisms contribute to the silencing of RIPed sequences in *Neurospora crassa*. Nucleic Acids Research **32**: 4237-4243.

COGONI, C., 2001 Homology-dependent gene silencing mechanisms in fungi. Annual Review of Microbiology **55**: 381-406.

COGONI, C., J.T. IRELAN, M. SCHUMACHER, T. SCHMIDHAUSER, E.U. SELKER and G. MACINO, G. 1996 Transgene silencing of the *al-1* gene in vegetative cells

of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. EMBO Journal **15**: 3153-3163.

COGONI, C., and G. MACINO, 1999a Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature **399**: 166-169.

COGONI, C., and G. MACINO, 1999b Homology-dependent gene silencing in plants and fungi: a number of variations on the same theme. Current Opinion in Microbiology **2**: 657-662.

COGONI, C., and G. MACINO, 1999c Posttranscriptional gene silencing in Neurospora by a RecQ DNA helicase. Science **286**: 2342-2344.

COGONI, C., and G. MACINO, 2000 Post-transcriptional gene silencing across kingdoms. Current Opinion in Genetics and Development **10**: 638-643.

CONRAD, C., and R. RAUHUT, 2002 Ribonuclease III: new sense from nuisance. The International Journal of Biochemistry & Cell Biology **34**: 116-129.

CONSTANCIA, M., B. PICKARD, G. KELSEY and W. REIK, 1998 Imprinting mechanisms. Genome Research **8**: 881-900.

DALMAY, T., A. HAMILTON, S. RUDD, S. ANGELL, and D.C. BAULCOMBE, 2000 An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for post-transcriptional gene silencing mediated by a transgene but not by a virus, Cell **101**(5): 543-53.

DAVIS, R. H., and F. J. DE SERRES, 1970 Genetic and microbiological research techniques for *Neurospora crassa*, pp. 79-143 in *Metabolism of Amino Acids and Amines*, edited by S. P. COLOWICK and N. O. KAPLAN. Academic Press, New York.

DEHIO, C., and J. SCHELL, 1994 Identification of plant genetic loci involved in a posttranscriptional mechanism for meiotically reversible transgene silencing, Proceedings of the National Academy of Sciences USA **91**: 5538-5542.

ELBASHIR, S. M., W. LENECKEL and T. TUSCHL, 2001 RNA interference is mediated by 21- and 22- nucleotide RNAs. Genes and Development **15**: 188-200.

FAGARD, M., S. BOUTET, J.B. MOREL, C. BELLINI and H. VAUCVHERET, 2000 AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proceedings of the National Academy of Sciences USA **97**: 11650-54.

FIRE, A., S. XU, M.K. MONTGOMERY, S.A. KOSTAS, S.E. DRIVER and C.C. MELLO, 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391**: 806-811.

FORREST, E. C., C. COGONI and G. MACINO, 2004 The RNA-dependent RNA polymerase, QDE-1, is a rate-limiting factor in post-transcriptional gene silencing in *Neurospora crassa*. Nucleic Acids Research **32**: 2123-2128.

FULCI, V. and MACINO, G., 2007 Quelling: post-transcriptional gene silencing guided by small RNAs in *Neurospora crassa*. Current Opinion in Microbiology **10**:199-203.

GALAGAN, J. E., S. E. CALVO, K. A. BORKOVICH, E. U. SELKER, N. D. READ *et al.*, 2003 The genome sequence of the filamentous fungus *Neurospora crassa*. Nature **422**: 859-868.

GOLDONI, M., G. AZZALIN, G. MACINO and C. COGONI, 2004 Efficient gene silencing by expression of double stranded RNA in *Neurospora crassa*. Fungal Genetics and Biology **41**: 1016-1024.

GRIFFITHS, A. J. F., and A. M. DELANGE, 1978 Mutations of the *a* mating-type gene in *Neurospora crassa*. Genetics **88**: 239-254.

GRISHOK, A., H. TABARA and C.C. MELLO, 2000 Genetic requirements for inheritance of RNAi in *C. elegans*. Science **287**: 2494-2497.

GRISHOK, A., A. E. PASQUINELLI, D. CONTE, N. LI, S. PARRISH *et al.*, 2001 Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. Cell **106**: 23-34.

GUO, S., and K. KEMPHUES, 1995 *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell **81**: 611-620.

HAAG, J. R., D. W. LEE and R. ARAMAYO, 2003 A GATEWAY™ destination vector for high-throughput construction of *Neurospora crassa histidine-3* gene replacement plasmids. Fungal Genetics Newsletter **50**: 6-8.

HALL, I. M., G. D. SHANKARANARAYANA, K. NOMA, N. AYOUB, A. COHEN *et al.*, 2002 Establishment and maintenance of a heterochromatin domain. Science **297**: 2232-2237.

HALL, J. G., 1997 Genomic imprinting: nature and clinical relevance. Annual Review of Medicine **48**: 35-44.

HAMILTON, A J., and D. C. BAULCOMBE, 1999 A novel species of small antisense RNA in posttranscriptional gene silencing. Science **286**: 950-952.

HAMMOND, S M., S. BOETTCHER, A.C. CAUDY, R. KOBAYASHI and G.J. HANNON, 2001 Argonaute2, a link between genetic and biochemical analyses of RNAi. Science **293**: 1146-1150.

HAMMOND, S.M., E. BERNSTEIN, D. BEACH and G.J. HANNON, 2000 An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature **404**: 293-96.

HANNON, G.J., 2002 RNA Interference, Nature **418**: 244-251.

HANNON, G. J., and J. J. ROSSI, 2004 Unlocking the potential of the human genome with RNA interference. Nature **431**: 371-378.

HASSOLD, T., and P. HUNT, 2001 To err (meiotically) is human: the genesis of human aneuploidy. Nature Reviews Genetics **2**: 280-291.

HENIKOFF, S., E. A. GREENE, S. PIETROKOVSKI, P. BORK, T. K. ATTWOOD *et al.*, 1997 Gene families: the taxonomy of protein paralogs and chimeras. Science **278**: 609-614.

HENIKOFF, S., and L. COMAI, 1998 *Trans*-sensing effects: the ups and downs of being together. Cell **93**: 329-332.

HUTVAGNER, G., J. MCLACHLAN, A. E. PASQUINELLI, E. BALINT, T. TUSCHL *et al.*, 2001 A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science **293**: 834-838.

HUTVAGNER, G., and P. D. ZAMORE, 2002 RNAi: nature abhors a double-strand. Current Opinion in Genetics and Development **12**: 225-232.

KADOTANI, N., H. NAKAYASHIKI, Y. TOSA and S. MAYAMA, 2004 One of the two Dicer-like proteins in the filamentous fungi *Magnaporthe oryzae* genome is responsible for hairpin RNA-triggered RNA silencing and related small interfering RNA accumulation. Journal of Biological Chemistry **279**: 44467-44474.

KENNERDELL, J.R., S. YAMAGUCHI and R.W. CARTHEW, 2002 RNAi is activated during *Drosophila* oocyte maturation in a manner dependent on *aubergine* and *spindle-E*. Genes and Development **16**:1884-89.

KENNERDELL, J.R., and R. W. CARTHEW, 1998 Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. Cell **95**: 1017-1026.

KNIGHT, S.W., and B. L. BASS, 2001 A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*. Science **293**: 2269-2271.

KUTIL, B. L., K. Y. SEONG and R. ARAMAYO, 2003 Unpaired genes do not silence their paired neighbors. Current Genetics **43**: 425-432.

LALANDE, M., 1996 Parental imprinting and human disease. Annual Review of Genetics **30**: 173-195.

LASALLE, J. M., and M. LALANDE, 1996 Homologous association of oppositely imprinted chromosomal domains. Science **272**: 725-728.

LEE, D. W., J. R. HAAG and R. ARAMAYO, 2003a Construction of strains for rapid homokaryon purification after integration of constructs at the *histidine-3* (*his-3*) locus of *Neurospora crassa*. Current Genetics **43**: 17-23.

LEE, D. W., R. J. PRATT, M. MCLAUGHLIN and R. ARAMAYO, 2003b An argonaute-like protein is required for meiotic silencing. Genetics **164**: 821-828.

LEE, D. W., K.-Y. SEONG, R. J. PRATT, K. BAKER and R. ARAMAYO, 2004 Properties of unpaired DNA required for efficient silencing in *Neurospora crassa*. Genetics **167**: 131-150.

LINGEL, A., B. SIMON, E. IZAURRALDE and M. SATTLER, 2004 Nucleic acid 3'-end.recognition by the Argonaute2 PAZ domain. Nature Structural Molecular Biology **11**: 576-77.

LINGEL, A., B. SIMON, E. IZAURRALDE and M. SATTLER, 2003 Structure and nucleic-acid binding of the *Drosophila* Argonaute 2 PAZ domain. Nature **426**: 465-469.

LIPPMAN, Z., and R. MARTIENSSEN, 2004 The role of RNA interference in heterochromatic silencing. Nature **431**: 364-370.

LIU, Q., T.A. RAND, S. KALIDAS, F. DU, H.E. KIM, *et al.*, 2003 R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. Science **301**: 1921-1925.

MA, J.B., K. YE and D.J. PATEL, 2004 Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Nature **429**: 318-22.

MACRAE, I., K. ZHOU, F. LI A. REPIC, A. BROOKS, W. CANDE, P. ADAMS and J. DOUDNA, 2006 Structural basis for double-stranded RNA processing by Dicer. Science **311**(5358): 195-198.

MARCOTTE, E. M., M. PELLEGRINI, H. L. NG, D. W. RICE, T. O. YEATES *et al.*, 1999 Detecting protein function and protein-protein interactions from genome sequences. Science **285**: 751-753.

MARTINEZ, J., A. PATKANIOWSKA, H. URLAUB, R. LURHMANN and T. TUSCHL, 2002 Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell **110**:563-74.

MATZKE, M. A., and J. A. BIRCHLER, 2005 RNAi-mediated pathways in the nucleus. Nature Reviews Genetics **6**: 24-35.

MATZKE, M., M. F. METTE, J. JAKOWITSCH, T. KANNO, E. A. MOSCONE *et al.*, 2001 A test for transvection in plants: DNA pairing may lead to *trans*-activation or silencing of complex heteroalleles in tobacco. Genetics **158**: 451-461.

MEISTER, G., and T. TUSCHL, 2004 Mechanisms of gene silencing by double-stranded RNA. Nature **431**: 343-349.

MELLO, C. C., and D. CONTE, JR., 2004 Revealing the world of RNA interference. Nature **431**: 338-342.

MIAN, I.S., 1997 Comparative sequence analysis of ribonucleases HII, III, PH, and D. Nucleic Acids Research **25**: 3187-3195.

MOCHIZUKI, K., and M. A. GOROVSKY, 2005 A Dicer-like protein in *Tetrahymena* has distinct functions in genome rearrangement, chromosome segregation, and meiotic prophase. Genes and Development 19: 77-89.

MOORE, M. J., 2002 Nuclear RNA turnover. Cell **108**: 431-434.

MOURRAIN, P., C. BECLIN, T. ELMAYAN, F. FEUERBACH, C. GODON, J.B. MOREL, D. JOUETTE, A.M. LACOMBE, S. NIKIC, N. PICAULT, K. REMOUE, M. SANIAL, T.A. VO and H. VAUCHERET, 2000 *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell **101**(5): 533-42.

NAPOLI C, C. LEMIEUX and R. JORGENSEN, 1990 Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell **2**: 279-289

NICHOLSON, R.H., and A.W. NICHOLSON, 2002 Molecular characterization of a mouse cDNA encoding Dicer, a ribonuclease III ortholog involved in RNA interference, Mammalian Genome **13**: 67-73.

NYKANEN, A., B. HALEY and P. D. ZAMORE, 2001 ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell **107**: 309-321.

PAL-BHADRA, M., U. PHADRA and J.A. BIRCHLER, 2002 RNAi-related mechanism affects both transcriptional and posttranscriptional transgene silencing in *Drosophila*. Molecular Cell **9**:315-27.

PERKINS, D. D., 1984 Advantages of using the inactive-mating type a^{m1} strain as a helper component in heterokaryons. Neurospora Newsletter **31**: 41-42.

PHAM, J.W. and E.J. SONTHEIMER, 2005 Molecular requirements for RNA-induced silencing complex assembly in the *Drosophila* RNA interference pathway. Journal of Biological Chemistry **280**(47): 39278-83.

PHAM, J .W., J.L. PELLINO, Y.S. LEE, R.W. CARTHEW, and E.J. SONTHEIMER, 2004 A Dicer-2-dependent 80S complex cleaves targeted mRNAs during RNAi in *Drospohila*. Cell **117**: 83-94.

PICKFORD, A. S., C. CATALANOTTO, C. COGONI and G. MACINO, 2002 Quelling in *Neurospora crassa*. Advances in Genetics **46**: 277-303.

PIRROTTA, V., 1999 Transvection and chromosomal *trans*-interaction effects. Biochimica et Biophysica Acta **1424**: M1-M8.

PLASTERK, R. H., 2002 RNA silencing: the genome's immune system. Science **296**: 1263-1265.

PRATT, R. J., and R. ARAMAYO, 2002 Improving the efficiency of gene replacements in *Neurospora crassa*: a first step towards a large-scale functional genomics project. Fungal Genetics and Biology **37**: 56-71.

PRATT, R. J., D. W. LEE and R. ARAMAYO, 2004 DNA methylation affects meiotic trans-sensing, not meiotic silencing, in *Neurospora*. Genetics **168**: 1925-1935.

RAJU, N. B., 1980 Meiosis and ascospore genesis in Neurospora. European Journal of Cell Biology **23**: 208-223.

RAJU, N. B., 1992 Genetic control of the sexual cycle in Neurospora. Mycological Research **96**: 241-262.

RIESSELMANN, L., and T. HAAF, 1999 Preferential S-phase pairing of the imprinted region on distal mouse chromosome 7. Cytogenetics and Cell Genetics **86**: 39-42.

ROMANO, N. and G. MACINO, 1992 Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. Molecular Microbiology **6**: 3343-3353

ROTONDO, G., and D. FRENDEWEY, 1996 Purification and characterization of the Pac1 ribonuclease of *Schizosaccharomyces pombe*. Nucleic Acids Research **23**: 2377-2386.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS (Editors), 1989 *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SAUNDERS, L.R. and G.N. BARBER, 2003 The dsRNA protein binding family: critical roles, diverse cellular functions, The FASEB Journal, **17**: 961-983.

SHIU, P. K., and R. L. METZENBERG, 2002 Meiotic silencing by unpaired DNA. Properties, regulation and suppression. Genetics **161**: 1483-1495.

SHIU, P. K. T., B. N. RAJU, D. ZICKLER and R. METZENBERG, 2001 Meiotic silencing by unpaired DNA. Cell **107**: 905-916.

SIJEN, T., J. FLEENOR, F. SIMMER, K. L. THIJSSEN, S. PARRISH *et al.*, 2001 On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107**: 465-476.

SINGER, M.J., B.A. MARCOTTE and E.U. SELKER, 1995 DNA methylation associated with repeat-induced point mutation in *Neurospora crassa*. Molecular Cell Biology **15**(10); 5586-5597.

SMARDON, A.M., J.M. SPOERKE, S.C. STACEY, M.E. KLEIN, N. MACKIN and E.M. MAINE, 2000 EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. Current Biology **10**:169-187.

SMITH, C.J.S., C.F. WATSON, C.R. BIRD, J. RAY, W. SCHUCH and D. GRIERSON, 1990 Expression of a truncated tomato polygalacturonase gene inhibits

expression of the endogenous gene in transgenic plants. Molecular Gene and Genetics **224**: 477-481.

SONG, J. J., J. LIU, N. H. TOLIA, J. SCHNEIDERMAN, S. K. SMITH *et al.*, 2003 The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nature Structural Biology **10**: 1026-1032.

SRB, A. M., M. BASL, M. BOBST and J. V. LEARY, 1973 Mutations in *Neurospora crassa* affecting ascus and ascospore development. Journal of Heredity **64**: 242-246.

TABARA, H., E. YIGIT, H. SIOMI and C. C. MELLO, 2002 The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in *C. elegans*. Cell **109**: 861-871.

TAHBAZ, N., F. A. KOLB, H. ZHANG, K. JARONCZYK, 23W. FILIPOWICZ *et al.*, 2004 Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer. EMBO Reports **5**: 189-1894.

TIJSTERMAN, M., K.L. OKIHARA, K. THIJSSEN and R.H. PLASTERK, 2002 PPW-1, a PAZ/PIWI protein required for efficient germline RNAi, is defective in a natural isolate of *C. elegans*. Current Biology **12**:1535-40.

TURNER, J. M., S. K. MAHADEVAIAH, O. FERNANDEZ-CAPETILLO, A. NUSSENZWEIG, X. XU *et al.*, 2005 Silencing of unsynapsed meiotic chromosomes in the mouse. Nature Genetics **37**: 41-47.

VAN DER KROL, A.R., L.A. MUR, M. BELD, J.N.M. MOL, and A.R. STUITJE, 1990 Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2**: 291-299

VOLPE, T. A., C. KIDNER, I. M. HALL, G. TENG, S. I. GREWAL *et al.*, 2002 Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science **297**: 1833-1837.

WATERHOUSE, P. M., M. B. WANG and T. LOUGH, 2001 Gene silencing as an adaptive defence against viruses. Nature **411**: 834-842.

WARGELIUS, A., S. ELLINGSEN, and A. FJOSE, 1999 Double-stranded RNA induces specific developmental defects in zebrafish embryos, Biochemistry and Biophysics Research Communications **263**: 156-161.

WIENHOLDS, E., M.J. KOUDIJS, F.J.M.VAN EEDEN, E. CUPPEN and R.H.A. PLASTERK, 2003 The microRNA-producing enzyme Dicer1 is essential for zebrafish development. Nature Genetics **35**: 217-218.

WILLIAMS, R.W. and G.M. RUBIN, 2002 *ARGONAUTE1* is required for efficient RNA interference in *Drosophila* embryos. Proceedings of the National Academy of Sciences USA **99**: 6889-94.

WU, C. T., and J. R. MORRIS, 1999 Transvection and other homology effects. Current Opinion in Genetics and Development **9**: 237-246.

XIE, Z., K.D. KASSCHAU, and J.C. CARRINGTON, 2003 Negative feedback regulation of Dicer-like1 in *Arabidopsis* by microRNA-guided mRNA degradation. Current Biology **13**: 784-789.

XIE, Z., L.K. JOHANSEN, A.M. GUSTAFSON, K.D. KASSCHAU, A.D. LELLIS, D. ZILBERMAN, S.E. JACOBSEN, J.C. CARRINGTON, 2004 Genetic and functional diversification of small RNA pathways in plants. PLoS Biology **2**(5): e104.

YAN, K. S., S. YAN, A. FAROOQ, A. HAN, L. ZENG *et al.*, 2003 Structure and conserved RNA binding of the PAZ domain. Nature **426**: 468-474.

ZAMORE, P. D., 2002 Ancient pathways programmed by small RNAs. Science **296**: 1265-1269.

ZAMORE, P.D., T. TUSCHL, P.A. SHARP AND D.P. BARTEL, 2000 RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101**: 25-33.

ZAMORE, P. D., 2002 Ancient pathways programmed by small RNAs. Science **296**: 1265-1269

VITA

Malcolm Thomas McLaughlin: 111 Fortnightly Boulevard, Herndon, Virginia 20170. Currently working as an analyst for the National Geospatial-Intelligence Agency (NGA) in Washington, D.C.

Education:

Bachelor of Arts – Spanish (*Cum Laude*), University of Southern California 1992

Juris Doctor – Law (*High Honors*), Chicago-Kent College of Law 1995

Bachelor of Science – Microbiology (*Cum Laude*), Texas A&M University 2001

Master of Science – Biology, Texas A&M University 2007.

Publications:

LEE, D. W., R. J. PRATT, M. MCLAUGHLIN and R. ARAMAYO, 2003b An argonaute-like protein is required for meiotic silencing. Genetics **164**: 821-828.